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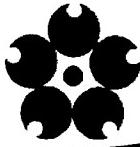


The Third International Meeting
on the
Molecular Genetics
and
Pathogenesis
of the
Clostridia



June 8 - 11, 2000
Kazusa Akademia Center
Chiba, Japan

Program and Abstracts



Supported by the Commemorative Association for the
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Letters from the Organizing Committee

Dear Colleagues,

The Organizing Committee has the pleasure to welcome you to the Third International Meeting on the Molecular Genetics and Pathogenesis of the Clostridia.

The location of the meeting, a tranquil area near metropolitan Tokyo, is an ideal setting for what we consider to be an exciting and stimulating program. We have succeeded in attracting the leading scientists to ensure that the conference focuses on the latest developments in the field.

We hope that the Symposium will provide an opportunity for you to meet old friends, make new acquaintances, and enjoy an academic atmosphere of Kazusa Akademia Park.

With best wishes,

S. Cole	H. Hayashi	B. McClane
S. Nakamura	J. Rood	R. Titball
R. Tweten		

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Scientific Program

Thursday, June 8th

- 15:00 Registration
17:00 Meeting of the IAC
18:00 Opening

Keynote Address

- 18:15 **S. Nakamura** (Kanazawa University, Japan)
Clostridium difficile and its disease in Japan

Friday, June 9th

Session 1: General Biology, Human and Veterinary Disease

Chair: Shinichi Nakamura

- 08:30 **L. Sonenshein**, B. Dupuy, N. Mani, J. Haraldsen (Tufts University, USA and Institut Pasteur, France): The relationship of stationary phase and sporulation to pathogenicity in *Clostridium difficile*.

- 08:50 **S. Karlsson**, L. Norin, L.G. Burman, T. Åkerlund (Swedish Institute for Infectious Disease Control, Sweden and Karolinska Institute, Sweden): Toxin expression by *Clostridium difficile* is regulated by cysteine and co-regulated with metabolic enzymes involved in butyric acid/butanol synthesis

- 09:10 **K. Mukherjee**, T. Åkerlund, L.G. Burman (Swedish Institute for Infectious Disease Control, Sweden): Analysis of extracellular proteins produced by *Clostridium difficile* using two-dimensional electrophoresis

- 09:30 **R. Labbe** (University of Massachusetts, USA): Physiology of sporulation of *Clostridium perfringens*

09:50 **A. Bentacor**, M.R. Fermepin, A. Lerea, R. de Torres (Universidad de Buenos Aires, Argentina): Relationship between sporulation and ϵ -toxin expression of *Clostridium perfringens* type D

10:10 **M. Nevas**, S. Hielm, M. Lindström, H. Korleala (University of Helsinki, Finland): Prevalence of *Clostridium botulinum* types A and B in honey samples detected by polymerase chain reaction

10:30 Coffee break

Session 2: Genome Organization and Molecular Genetics

Chair: Akinobu Okabe

11:00 **T. Shimizu** (University of Tsukuba, Japan): Genome Project of *Clostridium perfringens*

11:30 **S. Brynestad**, M.R. Sarker, B.A. McClane, P.E. Granum, J.I. Rood (The Norwegian School of Veterinary Science, Norway, University of Pittsburgh, USA and Monash University, Australia): Evidence that the *cpe*-containing plasmid can be transferred by conjugation to *cpe*-negative *C. perfringens* isolates

11:50 **J. Rood**, D. Lyras, V. Adams, J. Kim (Monash University, Australia): TnpX resolvase-mediated transposition of the clostridial Tn4451/Tn4453 family

12:10 **B. Wren** (London School of Hygiene and Tropical Medicine, UK): The *Clostridium difficile* 630 genome project

12:40 **C. von-Eichel-Streiber**, D.E. Mahony, V. Braun (Johannes Gutenberg-Universität, Germany and Dalhousie University, Canada): Characterization of a chimeric ribozyme integrated in the toxin A gene of *Clostridium difficile* strain C-34

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- 13:00 Lunch
14:00 Visit the Kazusa DNA Research Institute and plenary research discussion
16:00 Poster Session A: topics 1-4

Session 3: Membrane Active Toxins and Enzymes

Chair: Julian Rood

- 17:30 **R. Titball**, C.E. Naylor, D.S. Moss, J. Miller, A.K. Basak (DERA Porton Down, UK and Birkbeck College, UK): Crystal structure of *Clostridium perfringens* alpha-toxin
- 17:50 S. Ochi, M. Nagahama, H. Matsuda, **J. Sakurai** (Tokushima Bunri University, Japan): *Clostridium perfringens* alpha-toxin activates sphingomyelin metabolism system in sheep erythrocytes
- 18:10 **R.K. Tweten**, L. Shepard, A. Heuck, E. Hotze, M. Parker, J. Rossjohn, A.E. Johnson (University of Oklahoma, USA, Texas A&M University, USA, and St Vincent's Institute of Medical Research, Australia): Mechanism of membrane insertion of *C. perfringens* theta-toxin.
- 18:30 **N. Sugimoto** (Osaka University, Japan): Pathophysiology of the intoxication with *C. septicum* alpha-toxin in rats
- 18:50 **K.P. Song**, K.S. Tan, B.Y. Wee, S.Y. Chang (National University of Singapore, Singapore): Cloning, expression and characterization of TcdE protein from *Clostridium difficile*
- 19:10 **S. Dineen**, M. Bradshaw, E.A. Johnson (University of Wisconsin-Madison, USA): Isolation and characterization of a novel bacteriocin from *Clostridium botulinum* strain 213B
- 19:40 Dinner

Saturday, June 10th

Session 4: Neurotoxins

Chair: Bruce McClane

08:30 **K. Aktories** (University of Freiburg, Germany):
Clostridial toxins as tools to study cell biology

08:50 **S. Kozaki** (Osaka Prefecture University, Japan):
Botulinum toxin receptors

09:10 **K. Oguma**, Y. Fujinaga, K. Inoue, H. Arimitsu, Y. Sakaguchi, K. Yokota, N. Mahmut, T. Watanabe, T. Ohyama, K. Takeshi (Okayama University, Japan, University of Agriculture, Japan, and Hokkaido Institute of Public Health, Japan): Binding of *Clostridium botulinum* progenitor toxins to intestinal microvilli and erythrocytes

09:30 **M.A. Hanson**, R.C. Stevens (The Scripps Research Institute, USA): The product bound structure of botulinum neurotoxin type B catalytic domain

09:50 **N.F. Fairweather**, K.A. Sinha, M. Box, F. Ialli, G. Schiavo, H. Schneider, M. Groves, G. Siligardi, D. Fontinou, P. Emsley, N. Isaacs (Imperial College, Imperial Cancer Research Fund, London University College, Kings College, and University of Glasgow, UK): Mutants of tetanus toxin Hc fragment altered in biological activity coincide with protein-sugar structural data

10:10 **T. Karasawa**, X. Wang, T. Maegawa, S. Kozaki, Y. Gyobu, K. Yamakawa, H. Kato, S. Nakamura (Kanazawa University, Japan): Molecular epidemiology of type E botulinum toxin (BoNT/E)-producing *Clostridium butyricum*

10:30 Coffee break

Session 5: Enterotoxins

Chair: Nigel Minton

11:00 **J. Katahira** (Osaka University, Japan): Molecular cloning and functional characterization of receptors for *Clostridium perfringens* enterotoxin

11:25 **B. McClane**, U. Singh, C.M. Van Itallie, L.L. Mitic, J.M. Anderson (University of Pittsburgh, USA and Yale University, USA): Treatment of caco-2 cells with *Clostridium perfringens* enterotoxin induces the formation of multiple species of large complex, one of which contains the tight junction protein 'occludin'

11:50 **B. Stiles**, M. Hale, J.-C Marvaud, M. Popoff (USAMRIID, USA and Institut Pasteur, France): *Clostridium perfringens* iota toxin: receptor binding studies by fluorescence activated cytometry

12:10 **M. Thelestam**, E. Chaves-Olarte (Karolinska Institutet, Sweden): *C. difficile* toxins

12:35 **K. Aktories**, H. Barth, D. Blöcker, M. Eckhardt (University of Freiburg, Germany): Binding and uptake of *Clostridium botulinum* C2 toxin

13:00 Lunch

13:40 Sightseeing to Umi-hotaru (Tokyo Bay Aqua Line) and plenary research discussions

15:00 Poster Session B: topics 5-8

Session 6: Host-Pathogen Interactions

Chair: Klaus Aktories

16:30 **T. Karjalainen** (University of Paris-Sud, France): Characterization of *Clostridium difficile* adhesins

17:00 **M.R. Sarker**, R.J. Carman, B.A. McClane (University of Pittsburgh, USA and TechLab Inc., USA): The role of *Clostridium perfringens* enterotoxin (*cpe*) in *cpe*-associated gastrointestinal diseases

17:30 **A.E. Bryant**, D.L. Stevens (Veterans Affairs Medical Center, USA): The role of *plc*-induced activation of platelet gpIIb/IIIa in clostridial myonecrosis

17:50 **F. A. Uzal**, W.R. Kelly, W.E. Morris (The National Institute of Agricultural Technology, Argentina): Effect of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves

18:10 **S. Kamiya**, M. Takahashi, H. Yamaguchi, H. Taguchi, S. Nakamura (Kyorin University, Japan, Miyarisan Pharmaceutical Co. Ltd., Japan and Kanazawa University, Japan): Preventive effect of *Clostridium butyricum* on enterohemorrhagic *Escherichia coli* O157:H7 infection

19:00-late Banquet

Sunday, June 11th

Session 7: Regulation of Virulence

Chair: Linc Sonenshein

08:30 **B. Dupuy**, N. Mani, S.T. Cole, A.L. Sonenshein (Institut Pasteur, France and Tufts University, USA): TxeR, an RNA polymerase sigma factor that activates transcription of toxin genes of *Clostridium difficile*

09:00 **M.R. Popoff**, J.C. Marvaud, S. Raffestin (Institut Pasteur, France): Regulation of the toxinogenesis in *Clostridium botulinum* and *Clostridium tetani*

09:30 **S.S. Dineen**, M. Bradshaw, E.A. Anderson (University of Wisconsin-Madison, USA): Regulation of expression of type A botulinal neurotoxin

09:50 **J. Rood**, J. Cheung, S. McGowan, M. Bateman, M. Awad (Monash University, Australia): Functional analysis of the VirR response regulator from *Clostridium perfringens*

10:10 **T. Shimizu**, H. Yaguchi, K. Ohtani, T. Shimizu, H. Hayashi (University of Tsukuba, Japan): A novel RNA transcriptional regulator that controls toxin production in *Clostridium perfringens*

10:30 Coffee break

Session 8: Prophylaxis, Therapy and Diagnosis

Chair: Monica Thelestam

11:00 **T.V. Riley** (The University of Western Australia, Australia): The laboratory diagnosis of *Clostridium difficile* associated diarrhoea • Is it time for a change?

11:20 **L.C. Hunter**, I.R. Poxton (Edinburgh University, UK): Differentiation between *Clostridium botulinum* type C/D and *Clostridium novyi* type A by Western blot of surface antigens - Application to the identification of veterinary isolates from cases of dysautonomia

11:40 **J.L. Holley**, K.F. Griffin, E.D. Williamson, H. Garmory, J.E. Eyles, H.O. Alpar, S. Foynes, N.F. Fairweather, R.W. Titball (DERA Porton Down, UK, Aston University, UK, and University of London, UK): A comparison of vaccine delivery mechanisms for a *Clostridium botulinum* type F toxin sub-unit vaccine

12:00 **N. Minton** (Centre for Applied Microbiology & Research, UK): Tumour-specific delivery of anti-cancer agents using the spores of genetically modified Clostridia

12:20 **S. C. Liu**, T. Shibata, A.J. Giaccia, N.P. Minton, J.M. Brown (Stanford University, USA)

Genetically engineered Clostridia expressing a prodrug activating enzyme for cancer gene therapy

12:40 **G.C. Clark**, J. Miller, D.S. Moss, R.W. Titball (CBD Porton Down, UK and Birkbeck College, UK): The lysis of commercially available anti-cancer liposomes by *Clostridium perfringens* alpha-toxin

13:00 Lunch and Closing

Poster Sessions

Session 1

1. K. Jalava, T. Kaipainen, H. Eisgrüber, B. Schalch, A. Stolle, A. Heikinheimo, H. Korkeala: Majority of *Clostridium perfringens* strains isolated from food are *cpe*-negative
2. R. Labbe, Y.T. Lin: Isolation of *Clostridium perfringens* from U.S. retail foods
3. M. Lindström, S. Hielm, M. Nevas, H. Korkeala: Whitefish roe as a vehicle for type E botulism in Finland
4. S. Makino, Y. Chen, S. Miyata, K. Urakami, S. Shimamoto, R. Moriyama: Germination-specific spore cortex lytic enzymes from *C. perfringens* S40 spores: structure and regulation of enzymatic activity
5. J. McCoubrey, I.R. Poxton: Variation of the S-layer proteins of *Clostridium difficile*
6. G. Reysset, V. Briolat, C. Deschamps: Adaptive responses to oxidative stress of *C. perfringens*
7. M.R. Sarker, S. Sparks, R. Shivers, V.K. Juneja, B. McClane: Comparative studies of the effects if heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid versus chromosomal enterotoxin genes

Session 2

8. M. Elmore, T. O'Keefe, M. Bokori-Brown, A. Roberts, P. Mullany, N.P. Minton: Virulence factor discovery in *Clostridium difficile* through modulation of transcription factor expression using anti-sense RNA
9. J.D. Haraldsen, A.L. Sonenshein: Preliminary sequence analysis of RNA polymerase genes of *Clostridium difficile*

analysis of RNA polymerase genes of *Clostridium difficile*

10. P. Mullany, H. Wang: Demonstration that the site-specific recombinase TndX encoded by the conjugative transposon Tn5397 from *Clostridium difficile* is required and sufficient for integration and excision in *Escherichia coli*
11. P. Mullany, A. P. Roberts, V. Braun, C. von Eichel-Streiber: Demonstration of splicing of the group II intron present in the clostridial conjugative transposon Tn5397 and its role in conjugation
12. T. O'Keefe, M. Elmore, A. Ostrowski, A. McLeod, M. Bokori-Brown, N.P. Minton: Characterization of the specificity of the restriction/methylation system of *Clostridium difficile* and the development of shuttle vectors
13. S. Ohshima, K. Honjo, K. Ohtani, T. Shimizu, H. Hayashi, T. Shimizu: Complete nucleotide sequence of 54-kb virulence plasmid of *Clostridium perfringens* strain 13
14. Y. Sakaguchi, T. Hayashi, Y. Fujinaga, M. Ohnishi, K. Inoue, T. Murata, N. Mahmut, K. Nakayama, H. Arumitsu, K. Oguma: Genome structure of a botulinum toxin type C-converting phage, c-st
15. T. Shimizu, S. Ohshima, K. Ohtani, K. Honjo, H. Hayashi, T. Shimizu: Analysis of ten ribosomal RNA operons of *Clostridium perfringens* strain 13
16. T. Watanabe, Y. Sagane, H. Kouguchi, H. Sunagawa, K. Inoue, Y. Fujinaga, K. Oguma, T. Ohyama: Molecular composition of progenitor toxin produced by *Clostridium botulinum* type C strains 6813 and 6814
17. T. Yamagishi, M. Shimazu, Y. Hasegawa, M. Nakamura,

T. Maegawa, T. Karasawa, S. Nakamura: Diversity of nucleotide sequence of *Clostridium perfringens* epsilon-toxin gene

Session 3

18. M. Nagahama, S. Ochi, J. Sakurai: Role of the N- and C-domains of *Clostridium perfringens* alpha-toxin on biological activities

Session 4

19. H. Arimitsu, N. Mahmut, K. Inoue, Y. Fujinaga, K. Yokota, Y. Sakaguchi, T. Tsuji, K. Oguma: Prevention of absorption of the progenitor toxins from the small intestine by local immunity against the nontoxic component associated with *Clostridium botulinum* neurotoxins
20. K. Inoue, Y. Fujinaga, H. Arimitsu, Y. Sakaguchi, N. Mahmut, T. Watanabe, T. Ohya, K. Inoue, K. Oguma: Characterization of hemagglutinin activity of *C. botulinum* type A HA positive progenitor toxin

Session 5

21. J.-C. Marvaud, T. Smith, M. Hale, M. Popoff, B. Stiles, L. Smith: *Clostridium perfringens* iota toxin: mapping of receptor and Ia docking domains of Ib

Session 6

22. O. Matsushita, T. Koide, T. Toyoshima, A. Okabe: Substrate recognition of the collagen-binding domain of the *Clostridium histolyticum* class I collagenase

Session 7

-
- 23. R. Grau, J. Perez, L. Orsaria: Regulators of gene expression during stationary phase in *Clostridium perfringens*
 - 24. S. Katayama, O. Matsushita, S. Miyata, A. Okabe: Binding of the RNA polymerase α subunit to promoter upstream bent DNA of the *Clostridium perfringens* phospholipase C gene
 - 25. N. Mani, B. Dupuy, A.L. Sonenshein: Regulation of *Clostridium difficile* toxin genes by TxeR
 - 26. K. Ohtani, T. Shimizu, H. Hayashi, T. Shimizu: Virulence activating pheromone (VAP) of *Clostridium perfringens*
 - 27. K. Ohtani, T. Shimizu, H. Hayashi, T. Shimizu: Identification of a novel locus that controls toxin production in *Clostridium perfringens*

Session 8

- 28. T. Fukuda, M. Iwaki, T. Komiya, Y. Arakawa, M. Takahashi: Tetanus immunization: current status in Japan
- 29. T. Matsuki, K. Matsumoto, T. Takada, Y. Miyamoto, K. Watanabe, R. Tanaka: Development and evaluation of 16S rRNA-gene-targeted group-specific primers and probe for *Clostridium* cluster XIV in the human intestine
- 30. F.A. Uzal, P. Hugenholtz, S. Petray, L.L. Blackall, S. Moss, R.A. Assis, W.E. Morris: PCR detection of *Clostridium chauvoei*

Oral Presentation

CLOSTRIDIUM DIFFICILE AND ITS DISEASES IN JAPAN

S. Nakamura

Department of Bacteriology, School of Medicine, Kanazawa University,
Kanazawa, Japan

In 1977, Bartlett and Gorbach described in regard to the incidence of clindamycin-associated colitis that pseudomembranous colitis has not been noted in Japan despite extensive use of lincomycin for many years. This promoted us to pursue an epidemiological study of *Clostridium difficile* in Japan. In an early study (1981), we found that the carriage rate in healthy young Japanese adults is approximately 15%, distinctively higher than those in European countries which range between 0 and 2%. We also for the first time developed a serotyping method in relation to toxigenicity (1981). Delmee *et al* have followed our study by using a simple slide agglutination technique and confirmed our findings that there is a correlation between serogroup and toxigenicity. A recent finding regarding serogroups is that most strains belonging to serogroup F produce toxin B but not toxin A.

In the past decade many molecular-typing methods have been developed for epidemiological analysis of *C. difficile* infection. By these methods it has been shown that in some cases of nosocomial infection, a single outbreak strain is responsible. However, as to the carriage of *C. difficile* in healthy adults, it is unknown whether cross-infection occurs in communities and whether gut carriage is a permanent or temporary state. Focusing on these problems, we examined healthy adults in different communities for *C. difficile*. In this paper, we report the results of our molecular epidemiological analysis of *C. difficile* in different communities in comparison with epidemic strains in nosocomial infections of *C. difficile* in Japan.

THE RELATIONSHIP OF STATIONARY PHASE AND
SPORULATION TO PATHOGENICITY IN *CLOSTRIDIUM*
DIFFICILE. A.L. Sonenshein¹, B. Dupuy², N. Mani¹, and J.
Haraldsen¹. Tufts Univ. School of Medicine, Boston, MA, USA¹ and
Institut Pasteur, Paris, France².

Like most other bacteria, clostridial species react to nutrient limitation by inducing a variety of adaptive responses, most of which are designed to help the cell to search for and consume secondary nutritional sources. These responses include synthesis of toxins whose activity on eukaryotic cells may lead to death of competitors and release of potential nutrients. Prolonged nutrient limitation may lead to spore formation. In *C. difficile*, synthesis of toxins A and B is induced at the end of exponential growth phase in broth-containing media. This induction appears to have a nutritional component, since addition of glucose or other rapidly metabolizable sugars to the medium blocks toxin gene expression. Other physiological signals, such as sensing of high population density, may also contribute to toxin induction under some conditions. Our recent experiments indicate that growth-phase-dependent, glucose-sensitive regulation of toxin gene expression is mediated through TxeR, the product of the gene upstream of the *toxA-toxB* cluster and a positive regulator of transcription *in vivo* and *in vitro*. We have shown that TxeR acts as a sigma factor of RNA polymerase, activating the core RNA polymerase to bind to and transcribe from the *tox* promoters.

The fact that *C. difficile* is a spore-former could contribute in important ways to its pathogenicity. Spores may be the reservoir of disease-causing bacteria, both in the intestinal tract and in the environment, and may be responsible both for the onset of antibiotic-associated colitis and for relapse in treated patients. This model is being tested by introducing mutations into essential sporulation genes, such as those that encode sporulation-specific sigma factors.

**TOXIN EXPRESSION BY *CLOSTRIDIUM DIFFICILE* IS
REGULATED BY CYSTEINE AND CO-REGULATED WITH
METABOLIC ENZYMES INVOLVED IN BUTYRIC
ACID/BUTANOL SYNTHESIS**

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C. difficile strain VPI 10463 was grown in Peptone Yeast (PY) supplemented with amino acids, cysteine derivatives, butyric acid or butanol. Toxin levels were measured with EIA and intracellular proteins visualized by 2-D PAGE. Fatty acids and alcohols in *C. difficile* cultures were analyzed by gaschromatography. Promoter binding protein was purified by mixing cell extracts with magnetic beads (Dynabeads™) coated with PCR amplified promoter sequences of the genes of toxin A or B, respectively. Purified protein was visualized by SDS and 2-D PAGE and N-terminal sequenced.

Toxin production in PY was reduced 50 to 100-fold by addition of nine specific amino acids. The most potent amino acid in supressing toxin production was cysteine. Cysteine derivatives also had a similar effect. The expression of several metabolic enzymes were co-regulated with the toxins. One of these were identified as 3-hydroxybutyryl-CoA dehydrogenase (3-hbd), a key enzyme involved in the production of butyric acid/butanol in *C. acetobutylicum* (i.e. solventogenesis). Analysis of culture medium showed that butyric acid and to a lesser extent butanol accumulated in the medium during high toxin expression and addition of amino acids (i.e. cysteine) to the medium resulted in lower levels of these compounds. Toxin production was enhanced by adding butyric acid to the cultures and suppressed with butanol. Several proteins were identified to bind to the toxin promoters. One of the bound proteine were 3-hbd. Control experiments using DNA fragments representing other gene promoters or coding sequences are currently being tested to investigate the specificity of the DNA-protein interction.

ANALYSIS OF EXTRACELLULAR PROTEINS PRODUCED BY *CLOSTRIDIUM DIFFICILE* USING TWO-DIMENSIONAL ELECTROPHORESIS

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Clostridium difficile causes *C. difficile*-associated diarrhea and pseudomembranous colitis by releasing toxins A and B, whose mode of release or the regulation of their production is unclear. The aim of this study was to analyze extracellular proteins produced by *C. difficile* and to compare the protein patterns in media supporting high (PY) and low (PYG) toxin production by 1-D and 2-D PAGE. Proteins were subjected to N-terminal sequencing and subsequently identified through database searches.

In PY, toxins A and B were produced throughout the stationary phase in an approximately 2:1 ratio, which was 1:1 in the extracellular media. Toxin export was through secretion rather than lysis. Several PY specific proteins were identified having strong homologies to 3-oxoacyl-acyl carrier protein (24kDa), FixA (30kDa), FixB (45kDa), D-3-phosphoglycerate kinase (34kDa). The N-terminus of an extracellular protein of 36kDa abundant in both PY and PYG, showed a weak similarity to an open reading frame, ORF1, encoding a 79kDa protein in the *C. difficile* strain 630 genome database. Another 25kDa protein in the extracellular fraction in PYG, was also identified as a proteolytic fragment of ORF1. Two other proteins (50kDa and 55kDa) in this fraction showed similarity to a second ORF (ORF2) located adjacent to ORF1 on the chromosome. Both ORFs showed the highest similarity to N-acetyl-muramoyl-L-alanine amidase.

Results obtained indicate that toxins and multiple proteins involved in redox pathways are externalized concomitantly. Analysis of the regulation of such proteins will be an useful tool towards understanding the regulation of the *C. difficile* toxins.

PHYSIOLOGY OF SPORULATION OF CLOSTRIDIUM
PERFRINGENS, Labbe, R., University of Massachusetts, USA.

Bacterial sporulation is typically considered to result from a nutrient deprivation. As with Bacillus species sporulation of C. perfringens is divided into distinct morphological stages, with C. perfringens enterotoxin (CPE) appearing early in the process and released in large quantities upon sporangial autolysis. Rapidly metabolizable carbohydrates such as glucose readily inhibit the sporulation process. Therefore more complex carbohydrates, such as dextrin and starch, are used in sporulation media. Several amylolytic enzymes are excreted during vegetative cell growth and the presence and concentration of each changes with the onset of sporulation. There appears to be a correlation between enterotoxicity and amylase activity at 46°C. Of those examined only ent⁻ strains sporulated at 46°C using starch as the carbohydrate source though they sporulate at this temperature if raffinose is the principal carbohydrate. More than most spore-forming bacteria, sporulation of C. perfringens is highly medium- and strain-dependent. Both a metallo and serine protease are present intracellularly. The latter is sporulation-specific as determined by inhibition by protease-specific inhibitors, growth conditions (elevated temperatures) that inhibit sporulation, and the use of stage 0 sporulation mutants. In certain high CPE-producing strains an inclusion body is associated with sporulation. Its subunit composition indicates that it is composed of CPE whereas spore coat protein appears not to be composed of CPE, contrary to earlier reports.

RELATIONSHIP BETWEEN SPORULATION AND ϵ -TOXIN EXPRESSION
OF *CLOSTRIDIUM PERFRINGENS* TYPE D

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The kinetic of was investigated in relationship to the sporulation. The level of sporulation was evaluated by coloration and through determination of CFU ml⁻¹ in cultures submitted to thermal shock (70° 20'). The determination and detection of proteins was accomplished through electrophoresis (PAGE-SDS 15%) and Western blott. A vaccinal *C perfringens* type D with a high toxin level and sporulatory ability was used. Three different conditions were evaluated in order to establish relationship between sporulation and ϵ -toxin expression:

- 1) Time of the sporulation (1 to 10 days) in Duncan Strong broth
- 2) High concentration of inoculum (5 ml TGY pellet resuspended in 5 ml DS)
- 3) Thermal shock in a log-phase culture (TGY) and subsequent inoculation of washed spores in DS.

All the cultures were incubated in anaerobic jar gassed with 90 % H₂ and 10% CO₂ at 37°. The Western-blott indicates that the expression of ϵ -toxin levels in TGY are low. On the other hand the culture in DS (treat 1) determines a great toxin expression after 24 hs of development, it maintains high levels at 48 hs which then decreases jerkily. In coincidence, the first peak of sporulation is presented in 24 hs. The treatments 2 and 3 express intermediate level of toxin expressions in 24 hs, significantly greater than on a TGY culture.

PREVALENCE OF *CLOSTRIDIUM BOTULINUM*
TYPES A AND B IN HONEY SAMPLES DETECTED
BY POLYMERASE CHAIN REACTION

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A test protocol for detection of *Clostridium botulinum* type A and B spores in honey samples by polymerase chain reaction was developed. By using a centrifugation-membrane filtration method in prehandling of honey samples, we were able to detect as few as 0.1 spores per gram by the PCR after an incubation step. In this method honey samples of 25 g were diluted into 225 ml 1% Tween 80 solution in 65°C waterbath in 45 minutes and then centrifuged 8700-9000 x g for 30 min. Supernatant from every sample was filtered through 0.45 µm filter (Millipore) and the filter was inoculated into 9 ml TPGY broth for five days incubation in 30°C in anaerobic conditions. After the incubation 1 ml from every broth was transferred to a fresh 9 ml TPGY broth for 16 hours incubation in the same conditions. Of this suspension 1 ml was then washed and made into template for the PCR.

This method was used for prevalence study on *Clostridium botulinum* type A and B in 160 honey samples. Samples that gave positive results in PCR, were tested by biological assay to confirm the results. The percentage of positive samples was approximately 8 % in imported samples and of domestic samples about 5 % contained botulinal spores.

GENOME PROJECT OF *CLOSTRIDIUM PERFRINGENS*

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To investigate the genomic structure of *Clostridium perfringens*, we started a whole-genome-sequencing project on *C. perfringens* strain 13 that has been classified into type A. Chromosomal DNA from strain 13 was treated by sonication, and size fractionated fragments (1-2 kb) were ligated to SmaI site of pUC18. *Escherichia coli* transformed with the library were picked up and cultured in 96-well plates. The cultures were used as templates for PCR reaction, and the PCR products were subjected to direct sequencing using a M13 universal primer. Prior to the sequencing, a restriction and genetic map of the strain 13 genome was constructed using a pulsed field gel electrophoresis (PFGE). Its genome was found to consist of a 3.2-Mb chromosome and a 50-100 kb plasmid. Localization of 300 gene probes randomly selected from the sequencing templates was determined on strain 13 chromosome and plasmid. To date (version 9), a total of 49,556 templates has been sequenced (redundancy=8.0), and the sequences are assembled into 258 contigs covering 3,087,418 bases of the genome. In these contig sequences, we found several toxin genes that were previously reported, and interestingly, some candidates for genes encoding novel virulence factors were also identified. A reverse sequencing on selected templates and re-sequencing of ambiguous sequences are currently undertaken to complete the whole genome sequence of *C. perfringens*.

CONJUGATIVE TRANSFER OF THE *CPE* CONTAINING
VIRULENCE PLASMID FROM
C. PERFRINGENS

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The *Clostridium perfringens* enterotoxin (*CPE*) has been analysed by epidemiological, physical, and *cpe*-knockout studies as a critical virulence factor for the pathogenesis of *C. perfringens* type A food poisoning and several non-foodborne human gastrointestinal illnesses. The enterotoxin gene (*cpe*) is located on the chromosome of food poisoning isolates, but is on a plasmid in non-foodborne gastrointestinal disease and animal isolates. To evaluate the ability of the *cpe*- plasmid to transfer from *cpe*-positive isolates to *cpe*-negative isolates, a *C. perfringens* strain with a plasmid in which the *catP* gene was incorporated into the *cpe* gene (pMRS4969) was mated with several *cpe*-negative *C. perfringens* isolates. Chloramphenicol resistant transconjugants were selected and pMRS4969 was shown to transfer with a frequency of 10⁻² to 10⁻⁶ transconjugants per donor cell. The identity of the transconjugants was confirmed by PCR and by Southern hybridization analysis; all of the transconjugants tested showed the expected profiles. The plasmid present in the transconjugants was shown by PFGE and Southern hybridization analysis to be the same size as pMRS4969. This study provides the first direct evidence for virulence gene transfer in *C. perfringens* and identifies conjugation as the most likely transfer mechanism.

TnpX RESOLVASE-MEDIATED TRANSPOSITION OF THE CLOSTRIDIAL Tn4451/Tn4453 FAMILY

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The mobilisable transposons Tn4451 and Tn4453 mediate chloramphenicol resistance in *C. perfringens* and *C. difficile*, respectively. We have developed a sensitive transposition assay in *E. coli* and have shown that the transposon-encoded TnpX protein is responsible for excision of the element to form a non-replicating circular intermediate which then inserts into a target site that resembles the joint of that intermediate. Both processes involve a resolvase-mediated mechanism. The formation of the circular molecule joins the left and right ends of the element and leads to the formation of a functional promoter which contains a -35 box from the right end and a -10 box from the left end, as shown by primer extension and promoter probe studies. TnpX belongs to the resolvase family of site-specific recombinases but at 707aa it is much larger than most resolvases. It appears to have five major domains. The N-terminal domain has significant identity to classical resolvases, several of the other domains have potential coiled-coiled regions that may be involved in oligomerisation or DNA binding. Deletion analysis has shown that the 110aa C-terminal domain is not required for either excision or transposition and that domain 4 is essential for both activities. We also have designed a screening method for identifying random *tnpX* mutants. Seven point mutations that result in single aa substitutions have been identified, five of which are located in the resolvase domain. The remaining mutants are located in domain 2, providing evidence that this domain is also required for TnpX function.

THE *CLOSTRIDIUM DIFFICILE* 630 GENOME PROJECT

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Clostridium difficile is a major nosocomial pathogen world-wide causing disease ranging from antibiotic-associated diarrhoea to life-threatening pseudomembranous colitis. To understand this poorly studied problematic pathogen, the genome sequence of *C. difficile* strain 630 was initiated in June 99 in collaboration with staff at the Sanger Centre, Hinxton, UK.

The present assembly consists of 318 contigs over 1 Kb (213 contigs >2 Kb) totalling 4.250 Mb, and is assembled from 85,789 sequencing reads. The presentation will present preliminary analysis of sequence data. Initial analysis confirms the presence of toxins A and B and associated genes, a putative capsule with several sugar transferases, superoxide dismutase A and B, multi drug resistance loci, several transposon and IS elements, dozens of response regulators and a full flagellin assembly and chemotaxis system. The presence of these and other determinants will be related to the metabolism, survival and pathogenesis of *C. difficile*.

Using *Campylobacter jejuni* as an example, the presentation will demonstrate how DNA microarray technology can be applied for genomotyping. Such studies on *C. difficile* will be invaluable in studying genetic diversity of the organism and relating this to disease outcome.

Characterization of a chimeric ribozym integrated in the toxin A gene of *Clostridium difficile* strain C-34

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CdIS*t1*, a DNA insertion of 1975 bp, was identified within *tcdA-C34*, the enterotoxin gene of the *Clostridium difficile* isolate C34. Located in the catalytic domain A1-C34, CdIS*t1* combines features of two genetic elements. Within the first 434nt structures characteristic for group I introns were found; encoding the two transposase like proteins *tlpA* and *tlpB* nucleotides 435 to 1975 represent the remainder of a IS605-like insertion element. We show that the entire CdIS*t1* is accurately spliced from *tcdA1-C34* primary transcripts and that purified TcdA-C34 toxin is of regular size and catalytic activity. Search for CdIS*t1*-related sequences demonstrates that the element is widespread in toxinogenic and nontoxinogenic *C. difficile* strains, indicating mobility of CdIS*t1*. In strain C34 we characterise ten CdIS*t1* variants; all are highly homologous to CdIS*t1* (>93% identity), integrated in bacterial ORFs, show the typical composite structure of CdIS*t1* and are precisely spliced from their primary transcripts. CdIS*t1*-like chimeric ribozymes appear to combine the invasiveness of an insertion element with the splicing ability of a group I intron, rendering transposition harmless for the interrupted gene.

Crystal structure of *Clostridium perfringens* alpha-toxin

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The *Clostridium perfringens* alpha-toxin is the major virulence determinant in gas gangrene caused by *C. perfringens*. The toxin has phospholipase C activity and is haemolytic. Toxoid vaccines provide protection against disease in the murine model of infection. The crystal structure of the alpha-toxin from *Clostridium perfringens* reveals a two domain protein. The amino terminal domain contains the phospholipase C active site. Truncated forms of the alpha-toxin which lack the carboxy-terminal domain retain phospholipase C activity but are no longer haemolytic or toxic. The carboxy-terminal domain adopts a fold similar to that of C2-and C2-like domains from various eukaryotic phospholipid-binding proteins. We have identified three calcium-binding sites within the carboxy-terminal domain which are thought to play a role in membrane phospholipid recognition. The amino- and carboxy-terminal domains are joined by a flexible linker peptide. A loop which extends from the amino-terminal domain also makes contact with the carboxy-terminal domain. This loop region appears to be mobile – moving to allow access to the active site cleft when calcium ions are present. We have used this information to develop a model describing the interaction of the alpha-toxin with cell membrane phospholipids.

**CLOSTRIDIUM PERFRINGENS ALPHA-TOXIN ACTIVATES
SPHINGOMYELIN METABOLISM SYSTEM IN SHEEP
ERYTHROCYTES**

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We have suggested that the first step of alpha-toxin-induced hemolysis of rabbit erythrocytes is due to hydrolysis of phosphatidylcholine (PC) by the toxin and that the toxin-activated phosphatidic acid formation system is closely related to hemolysis of rabbit erythrocytes. Nelson reported that no PC is detected in the sheep erythrocytes and sphingomyelin (SM) accounted for about 50 % of the total phospholipids. Recently the activation of a SM cycle is recognized as a key event in the signal transduction cascade involved in cellular proliferation, differentiation and apoptosis. Therefore, we investigated the relationship between the toxin-induced hemolysis and SM metabolism system in sheep erythrocytes. The toxin induced the hot-cold hemolysis, and formation of ceramide and phosphorylcholine in the erythrocytes in a dose-dependent manner. N-oleoilethanolamine, ceramidase inhibitor, dose-dependently inhibited the toxin-induced hemolysis. Treatment of the cells with the toxin in the presence of this agent led to accumulation in ceramide, but no effect on phosphorylcholine level in the cells. *Threo*-dihydrosphingosine, sphingosine kinase inhibitor, strongly blocked the toxin-induced hemolysis and accumulated sphingosine in the cells in a dose-dependent manner. The toxin stimulated production of sphingosine-1-phosphate in the presence of sphingosine in the skinned cells. In addition, sphingosine 1-phosphate potentiated the toxin-induced hemolysis of the skinned cells. These observations suggest that alpha-toxin-induced hemolysis of sheep erythrocyte is closely related to activation of SM metabolism system.

MECHANISM OF MEMBRANE INSERTION OF *C. PERFRINGENS*
THETA TOXIN.

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Clostridium perfringens theta toxin (perfringolysin O or PFO) is a cholesterol dependent, pore-forming cytolysin. PFO belongs to a large family of *cholesterol-dependent cytolysins* (CDCs) which oligomerize into large supramolecular complexes, comprised of up to 50 monomers, which insert into and lyse cholesterol-containing membranes. Using a variety of fluorescence-based spectroscopic analyses we have identified two membrane-spanning beta hairpins (TMH1 and TMH2) per monomer which contribute the formation of a large, membrane-spanning beta barrel. These transmembrane hairpins (TMHs) are unusual for two reasons; they are derived from a total of six alpha helices in the soluble monomer and the fact that each monomer contains two transmembrane beta hairpins instead of a single beta hairpin as is found for other pore-forming toxins. We have also determined that PFO forms a large, relatively homogeneous, prepore complex on the membrane before the simultaneous insertion of the TMHs. This transition can be prevented by the introduction of a disulfide between TMH1 and domain 2 of PFO. This disulfide mutant of PFO is hemolytically inactive and cannot insert either TMH1 or THM2 into the membrane unless the disulfide is reduced. However, this disulfide-locked mutant can still assemble into the same oligomeric prepore complex as native toxin on the membrane surface. Therefore, the formation of a prepore complex accommodates the insertion of the large beta barrel of the CDCs.

Pathophysiology of the intoxication with *C. septicum* α toxin in rats

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Clostridium septicum is known as a gas-gangrene organism, and is the most frequent cause of non-traumatic clostridial gas gangrene. α toxin, a single polypeptide of a molecular weight of 49,800, is believed to be the main pathogenic factor produced by the organism. However, little is known about the mechanism of the lethal action of the toxin. To clarify *in vivo* target organ/tissues of the toxin, I investigate the pathophysiology of the intoxication with the toxin in rats. Rats injected intravenously with 20, 7, 2 μ g of purified α toxin ceased respiration 6 \pm 1, 12 \pm 4, 145 \pm 14 min after the injection, respectively. Changes in electrocardiogram are the most prominent ones among the changes in vital signs of the intoxicated animals. *Ex vivo* perfusion of the toxin to the isolated rat hearts elicited increase in coronary perfusion pressure and changes in electrocardiomyogram. The toxin caused cessation of spontaneous beats of the isolated right atrium. The results indicate that α toxin of *C. septicum* acts directly on the myocardium and produces myocardial contracture. It is likely that the cardiac dysfunction thus produced cause the circulatory disturbance, leading to the death of the intoxicated animals. Studies on the effects of α toxin on membrane excitability of myocardial cells are now in progress in my laboratory

CLONING, EXPRESSION AND CHARACTERIZATION OF TcdE PROTEIN FROM *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile is an anaerobe that has been implicated as the causative agent for pseudomembranous colitis. The virulence factors are located within a 19.6 Kbp pathogenicity locus (PaLoc) which encompasses two toxin genes (*tcdA* and *tcdB*) and at least three accessory genes (*tcdC*, *tcdD* and *tcdE*). One of the accessory genes, *tcdE*, is located between the two well characterized toxin genes, *tcdA* and *tcdB*. Although its nucleic acid sequence has been determined, its function and potential role in the pathogenicity of the bacteria is still not known. We report here the cloning, expression and characterization of *tcdE* in an *E. coli* host. Amino acid sequence analysis and hydrophobicity comparison of the putative protein showed it to be highly homologous to a class of autolytic proteins called holins. One of the known functions of holins is to lyse host cells for the release of progeny phages. When recombinant clone containing *tcdE* was induced, it caused the bacterial host cells to lyse, suggesting that TcdE may be cytotoxic. Controls including parental expression vector and host cell alone did not show any lysis. Dislodged membrane on the surface of the bacteria was observed with electron microscopy. All these conform to the structure and functional activities of holins. We therefore propose here that TcdE may be a holin-like protein.

ISOLATION AND CHARACTERIZATION OF A NOVEL
BACTERIOCIN FROM *CLOSTRIDIUM BOTULINUM*
STRAIN 213B

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A novel bacteriocin, boticin B, was found to be produced by *Clostridium botulinum* strain 213B. It is a heat-stable protein with a molecular mass of approx. 4002 Da. The bacteriocin has antimicrobial activity against Group I and II *C. botulinum* strains. The gene encoding the bacteriocin was cloned and sequenced. A mixed oligonucleotide (18-mer) homologous to an already known partial protein sequence of boticin B hybridized to a 3.0 kb HindIII fragment of an 18.8 kb plasmid from the *C. botulinum* strain 213B. The fragment was cloned into pBluescript KS II (+) and subcloned into a clostridial shuttle vector, pJIR1457. DNA sequencing revealed the boticin B structural gene to be an open reading frame encoding 50 amino acids. The plasmid pMVP1113 (pJIR1457 containing the 3.0 kb cloned fragment of the boticin B plasmid) was introduced into the *C. botulinum* strain 62A by conjugative transfer from the *E. coli* strain S17-1. The *C. botulinum* strain 62A(pMVP1113) transconjugant expressed boticin B, although at much lower levels than that observed in *C. botulinum* 213B. To our knowledge, this is the first demonstration of a bacteriocin from toxigenic Group I *C. botulinum*.

CLOSTRIDIAL TOXINS AS TOOLS TO STUDY CELL BIOLOGY

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Several clostridial toxins act on eukaryotic cells by modifying Rho GTPases, which are molecular switches in a large array of signal transduction processes and master regulators of the actin cytoskeleton. Among these toxins are the members of the family of large clostridial cytotoxins consisting of *C. difficile* toxins A and B, including various isoforms, *C. sordellii* lethal and hemorrhagic toxins and the α -toxin from *C. novyi*. These toxins catalyse the glucosylation or N-acetylglucosaminylation (α -toxin) of Rho GTPases using UDP-glucose or UDP-GlcNAc as a cosubstrate. The toxins differ in their substrate specificity. Whereas toxins A and B, the hemorrhagic toxin and the α -toxin modify all members of the Rho subfamily of small GTPases including Rho, Rac and Cdc42, *C. sordellii* lethal toxin and related toxin B isoforms glucosylate Rac (possibly Cdc42) but not Rho; in addition Ras subfamily members (e.g., Ras, Ral and Rap) are substrates for these toxins. Glucosylation of GTPases occurs at Thr37 of Rho (Thr35 of Rac or Ras). This modification inhibits the biological functions of Rho/Ras proteins by blocking the interaction of the GTPases with their effectors. Therefore, the different cytotoxins are specific and highly potent cell biological tools to study the involvement of Rho/Ras GTPases in various signaling pathways. In addition to glycosylating toxins, the ADP-ribosylating *C. botulinum* C3 transferase, which selectively modifies Rho but not Rac or Cdc42 is frequently used as a tool to inhibit Rho signaling. Because C3 is not able to enter cells readily, it is used as a fusion toxin attached to the N-terminal part of the enzyme component of the binary *C. botulinum* C2 toxin. Examples of the application of the various toxins in studies on signal transduction of eukaryotic cells will be presented.

BOTULINUM TOXIN RECEPTORS

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Clostridium botulinum neurotoxin (BoNT) exerts its toxin action by inhibition of neurotransmitter release and seven immunological types (A-G) have been described. Toxin action has been proposed to involve binding to receptors on the presynaptic membrane, but it is still obscure how BoNT recognizes specific component(s) on the surface of presynaptic membrane. Competition experiments with different types of BoNTs showed that they bind to type specific components. We have purified type B BoNT (BoNT/B) binding proteins from rat brain and identified them as synaptotagmins I and II. However, BoNT/B did not bind to synaptotagmin alone, but was only observed in the presence of ganglioside GT1b or GD1a, suggesting that synaptotagmin form the toxin binding site by associating with the specific gangliosides. Recombinant mutants of synaptotagmin II allowed us to demonstrate that the N-terminal domain retains BoNT/B binding activity. There were several reports that GT1b binds to BoNTs and causes loss of toxicity. In order to clarify its role in constituting as a component of receptor, we examined the inhibitory effect of a monoclonal antibody against GT1b on BoNT binding to receptor and toxic action to rat superior cervical ganglion neurons. The antibody antagonized the actions of both BoNT/A and BoNT/B. These data indicate that GT1b functions as a common and complementary components for BoNT receptors. Protein components like synaptotagmin appear to define type specificity of the BoNT receptor.

BINDING OF *CLOSTRIDIUM BOTULINUM* PROGENITOR TOXINS TO INTESTINAL MICROVILLI AND ERYTHROCYTES

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Clostridium botulinum neurotoxin (~150 kDa) associate with nontoxic components in the culture fluids to form larger complexes designated progenitor toxins. In the type A strain, three different sized progenitor toxins with molecular masses of 900 kDa (19S), 500 kDa (16S), and 300 kDa (12S) are produced. The type B, C, and D strains produce both the 16S and 12S toxins. The 12S toxin is formed by association of a neurotoxin with a nontoxic component having no hemagglutinin activity, while 19S and 16S toxins are formed by conjugation of the 12S toxin with a nontoxic component having HA activity (HA). We previously reported that type C HA plays an important role in the effective absorption from the small intestine of guinea pig, and that type A, B, C, and D HAs consist of four subcomponent designated HA1(~33 kDa), HA2 (~17 kDa), HA3a (~23 kDa), and HA3b (~53 kDa).

In this study, each subcomponent of type A and C HAs was produced as GST fusion proteins in *E. coli*. and analyzed its binding to intestinal epithelial cells and human erythrocytes. In both types, GST-HA2 and GST-HA3a showed no detectable binding to both epithelial cells and erythrocytes. On the other hands, GST-HA1 and HA3b of both types strongly bound to these cells. The receptors for HA1 and HA3b were seemed to be polysaccharides containing sialic acid and galactose, respectively. Type A HA positive progenitor toxin (16S and 19S toxins) mainly bound to polysaccharides containing galactose, and type C 16S toxin bound to those containing sialic acid. Therefore, it was concluded that type A HA positive progenitor toxin bound to their receptor via HA1, while type C 16S toxin bound via HA3b. Binding experiment using deletion mutants of HA1 and HA3b revealed that the C-terminal regions of both subcomponets are important for their binding.

THE PRODUCT BOUND STRUCTURE OF BOTULINUM NEUROTOXIN TYPE B CATALYTIC DOMAIN

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Synaptic vesicle membrane protein (synaptobrevin) is a member of the evolutionarily conserved SNARE proteins that are responsible for neurotransmitter vesicle docking to the neuronal cell membrane. Botulinum neurotoxin type B is a zinc protease that disrupts neurotransmitter release by selectively cleaving synaptobrevin. We report here the three-dimensional crystal structures of apo-botulinum neurotoxin serotype B catalytic domain or light chain (BoNT/B-LC) at 2.2 Å resolution, and the product-trapped synaptobrevin•BoNT/B-LC co-crystal structure at 1.9 Å resolution. The toxin remains inactive until the catalytic domain is removed from the rest of the toxin and enters into the neuronal cytoplasm. Comparison of the structures of the holotoxin with the separated light chain shows a rearrangement of the light chain active site loops upon separation that exposes the toxin active site and enables synaptic vesicle protein recognition. The synaptobrevin-toxin product bound structure provides clues to the exquisite specificity of the botulinum neurotoxins for their targets and the mechanism of proteolysis.

MUTANTS OF TETANUS TOXIN H_C FRAGMENT ALTERED IN BIOLOGICAL ACTIVITY COINCIDE WITH PROTEIN-SUGAR STRUCTURAL DATA

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Tetanus toxin binds neuronal tissue prior to internalisation and trafficking to the central nervous system. Binding of the carboxy terminal 50 kDa H_C fragment of tetanus toxin to polysialogangliosides is important for this initial cell binding step. From the three dimensional structure of H_C, a series of mutants were constructed to investigate the binding and uptake process. Mutant proteins were tested for binding to GT1b gangliosides, rat PC12 cells and primary motoneurons and for their ability to undergo retrograde transport in mice.

Two classes of mutant were obtained: (1) those containing deletions in loop regions within the C-terminal β trefoil domain which showed greatly reduced ganglioside and cell binding and did not undergo retrograde transport and (2) those that showed reduced ganglioside binding, but retained primary neuronal cell binding and retrograde transport. The second class included point mutants of Histidine-1293, previously shown to be involved in GT1b binding.

In collaboration, we have determined the crystal structure of Hc bound to sugars, which has identified several sugar binding sites within the β trefoil domain. These sugar binding sites are located at or near the sites of mutants which alter the binding and transport functions of Hc. This structural and functional data provides a insight to the mechanism of ganglioside binding and its importance in the biological activity of Hc fragment.

MOLECULAR EPIDEMIOLOGY OF TYPE E BOTULINUM TOXIN
(BoNT/E)-PRODUCING *CLOSTRIDIUM BUTYRICUM*

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BoNT/E-producing *C. butyricum* is one of the etiological agents of botulism. In this study, BoNT/E-producing *C. butyricum* strains isolated from botulism cases or soil specimens in Italy and in China were analyzed by using nucleotide sequencing of the *bont/E* gene, random amplified polymorphic DNA (RAPD) assay, pulsed-field gel electrophoresis (PFGE), and Southern blot hybridization for the *bont/E* gene. Nucleotide sequences of the *bont/E* gene of 11 Chinese isolates and of the Italian strain BL 6340 were determined. The nucleotide sequences of the *bont/E* gene of 11 *C. butyricum* isolates from China were identical. The deduced amino acid sequence of BoNT/E from the Chinese isolates showed 95.0% and 96.9% identity with those of BoNT/E from *C. butyricum* BL 6340 and from *Clostridium botulinum* type E, respectively. The BoNT/E-producing *C. butyricum* strains were divided into the following three clusters based on the results of RAPD assay, macrorestriction profiles by PFGE, and Southern blot hybridization: strains associated with infant botulism in Italy, strains associated with food-borne botulism in China, and isolates of soil specimens of the Weishan lake area in China. A DNA probe for the *bont/E* gene was hybridized with the nondigested chromosomal DNA of all toxigenic strains tested, indicating chromosomal localization of the *bont/E* gene in *C. butyricum*. The results suggest that the clonal distribution of BoNT/E-producing *C. butyricum* occurs over a vast area.

Molecular Cloning and Functional Characterization of Receptors for *Clostridium perfringens* Enterotoxin

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Clostridium perfringens enterotoxin (CPE), known as the causative agent of *C. perfringens* food poisoning, elicits fluid accumulation in the intestinal tract by altering the membrane permeability of intestinal epithelial cells. Pore formation in the cytoplasmic membrane is generally accepted as the underlying mechanism of this effect. As has long been believed, expression cloning demonstrated the existence of a specific cell surface receptor for CPE, which we named CPE-receptor. Sequence similarities and reconstitution experiments indicates that CPE-receptor is the fourth member of the Claudin family proteins that are the major constituents of the epithelial and endothelial tight junctions. The C-terminal receptor binding fragment of CPE binds to specific Claudins and removes them from the tight junction and as a result the tight junction permeability was downregulated. Though this phenomenon probably may not be the underlying mechanism of the symptoms associated with *C. perfringens* food poisoning, the C-terminal fragment of CPE proved to be a novel and useful reagent to specifically modulate the tight junctional barrier function. In contrast, the binding of intact CPE to cell surface via CPE-receptor/Claudin causes morphological alterations of the cell concomitant with formation of a high molecular weight complex. This complex may be a pore in the cytoplasmic membrane. Since the CPE-receptor was also involved in the complex, it may function in both target cell recognition and pore-formation. Further characterization of functional roles of CPE-receptor will provide a clue to understand the action mechanism of CPE.

TREATMENT OF CACO-2 CELLS WITH *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN INDUCES THE FORMATION OF MULTIPLE SPECIES OF LARGE COMPLEX, ONE OF WHICH CONTAINS THE TIGHT JUNCTION PROTEIN 'OCCLUDIN'

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Clostridium perfringens enterotoxin (CPE) is responsible for the symptoms of *C. perfringens* type A food poisoning, as well as symptoms of certain nonfoodborne gastrointestinal diseases. CPE apparently acts via a unique multi-step action. First, CPE binds to recent protein(s) in the plasma membrane, forming a small complex. This small complex then binds with another eucaryotic protein(s) to form large complex, which induces cytotoxicity by changing the membrane permeability of the plasma membrane. Previous affinity chromatography studies have identified two eucaryotic proteins (~45-50 and ~65-70 kDa) which appear to be associated with CPE in large complex. Additionally, Katahira *et al* showed in 1997 that certain Claudins, which are ~22kDa tight junction proteins, also appear to be present in large complex.

Our present study used Western immunoblot analysis to demonstrate that large complex formed in CPE-treated CaCo-2 (Colon Carcinoma) cells consists of multiple species, with sizes of ~200 kDa, ~155 kDa and ~135 kDa. The ~200 kDa large complex species was shown to contain Occludin, a ~65 kDa tight junction protein. Immunoprecipitation studies revealed that Occludin is not present in small complex, suggesting that Occludin associates with CPE late in CPE's action. This suggestion received further support when rat fibroblast transfectants expressing Occludin were shown not to bind CPE. Those Occludin transfectants were also insensitive to CPE, indicating that the presence of Occludin is insufficient to convey CPE's cytotoxic effect. Collectively, these results, 1) identify 'Occludin' as the ~65 kDa protein previously linked to large complex, 2) further strengthen associations between CPE and tight junction proteins, and 3) reveal that CPE action is more complicated than previously appreciated.

***Clostridium perfringens* Iota Toxin: Receptor Binding Studies with Fluorescence Activated Cytometry**

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The binding properties of *C. perfringens* type E iota toxin were studied by using Vero cells and flow cytometry. The binding component of iota toxin, iota b (Ib), bound to Vero cells within 10 min at 4, 25, or 37°C. Varying concentrations of Ib yielded a saturated dose effect that was inhibited by *C. perfringens* type E or *C. spiroforme* antisera. The protoxin form of Ib bound to the cell surface but trypsinization of Ib was necessary for docking of the ADP-ribosylating component, iota a (Ia). Ia attached to cell-bound Ib within 10 min at 37°C but surface levels of Ia had decreased ~90% after 30 min. Surface levels of Ib also decreased over time (80% by 120 min), thus suggesting release, internalization, or embedment into the membrane. Pretreatment of Vero cells with various glycosidases or lectins did not affect the binding of Ib. However, pretreatment with varying pronase concentrations effectively inhibited Ib binding to the cell surface. These results reveal the usefulness of flow cytometry for cell binding studies with bacterial toxins, like *C. perfringens* iota toxin.

C.DIFFICILE TOXINS

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The two major toxins implicated in *C.difficile* disease are toxin A (TcdA) and toxin B (TcdB). Traditionally TcdA is denoted as an enterotoxin and TcdB as a cytotoxin, but both appear important in the disease and both are cytotoxic. TcdA (MW 308 kDa) and TcdB (MW 269 kDa) belong to the family of Large Clostridial Cytotoxins (LCTs). Like other LCTs they have a repeat structure in the C-terminal domain which is responsible for binding to cells, and an enzymatically active N-terminal domain. In order to act on cells these toxins bind to specific (unidentified) receptors and are taken up by receptor-mediated endocytosis. Microinjection experiments demonstrate indirectly that the *C. difficile* toxins do not necessarily have to be activated upon cellular internalisation, although they might be intra-endosomally cleaved before translocating an active fragment into the cytosol. TcdA and TcdB are glucosyltransferases, i.e., they transfer glucose from the co-factor UDP-glucose to small GTPases of the Rho subfamily, thereby inactivating them. Cellular targets for both toxins are Rho, Rac and Cdc42. TcdA in addition glucosylates Rap. A major reason for the approximately 1000 fold lower cytotoxic potency of TcdA in most cell types is that its enzymatic potency is about 100-fold lower than that of TcdB. This suggests that glucosylation of small GTPases might not be the primary *in vivo* effect of TcdA. A new member of the LCT family was recently identified in a *C.difficile*-1470 and will be discussed.

BINDING AND UPTAKE OF *CLOSTRIDIUM BOTULINUM* C2 TOXIN

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The actin-ADP-ribosylating *Clostridium botulinum* C2 toxin is binary in structure and consists of two components, a binding component (C2II) and an enzyme component (C2I). For entry into target cells, C2II binds to a cell surface receptor and mediates uptake of C2I by receptor-mediated endocytosis. Here we studied the C2 toxin receptor binding and cellular uptake mechanisms.

We found that a recently identified Chinese hamster ovary (CHO) cell mutant (RK14) lacking a functional C2 receptor is deficient in N-acetylglucosaminyltransferase I (GlcNAc-TI), the key enzyme for initiating synthesis of hybrid and complex asparagine-linked glycans. Expression of a functional active GlcNAc-TI in RK14 cells made the cells again sensitive for C2 toxin. Activation of C2II by trypsin results in formation of SDS-resistant oligomers. Electron microscopy and analytical ultracentrifugation revealed heptamers of ~ 420 kDa. C2II oligomers seem to be pre pores which bind C2I. Bafilomycin A1 inhibited uptake of C2 toxin indicating that C2 toxin translocates from an acidic compartment into the cytosol. At pH < 5.6 of cell culture medium, C2 toxin entered the cytosol in the presence of baflomycin most likely by a direct translocation from outside the cell into the cytosol.

The data indicate that *Clostridium botulinum* C2 toxin forms heptamers to bind to asparagine-linked complex or hybrid carbohydrates, which serve as receptors for the toxin, subsequently C2 is taken up from an acidic compartment.

CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE ADHESINS

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The initiating signal for *C. difficile* proliferation in the gut is the destruction of the normal flora which may profoundly alter the gut microenvironment. We have investigated the role of various stress factors in the first step of gut colonization, i.e., cell attachment. The influence of the environmental stresses on *C. difficile* adhesion has been investigated *in vitro* using various cell lines.

The following stress conditions were found to increase cell attachment: oxygen, heat, osmotic and acid shock; and iron depletion. Depending of bacterial culture conditions, Vero cells could bind two or three bacterial surface proteins with a molecular weight of 70, 50 and 40 kDa.

Our laboratory has undertaken genetic characterization of the heat shock response in *C. difficile*. The following heat-shock proteins have been investigated: P27, Hsp60 (GroEL), Hsp70 (DnaK), and Cwp66 (66 kDa cell wall protein). The genes encoding Hsp60, Hsp70 and *cwp66* have been isolated, partially characterized and the corresponding proteins have been purified.

GroEL and Cwp66 appear to play a role in cell attachment as antibodies raised against GroEL and the C-terminal portion of purified Cwp66 were found to partially inhibit adherence to tissue culture cells. These proteins are surface-exposed. In the strain 630 a gene encoding an S-layer protein (adhesin) was found to be in close vicinity of the *cwp66* gene. The S layer, the Cwp66 and other proteins in the cluster share a common domain that could constitute a cell wall anchoring motif. Our results suggest that *C. difficile*, analogously to many other bacteria, possesses multiple surface-exposed adhesins.

THE ROLE OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN (CPE) IN CPE-ASSOCIATED GASTROINTESTINAL DISEASES.

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Epidemiologic evidence has implicated *Clostridium perfringens* enterotoxin (CPE) as a virulence factor in the pathogenesis of several gastrointestinal (GI) diseases caused by *C. perfringens* type A isolates, including *C. perfringens* type A food poisoning and nonfoodborne GI diseases, such as antibiotic-associated diarrhea and sporadic diarrhea.

In order to better evaluate the contribution of CPE to the GI pathogenesis of *cpe*-positive *C. perfringens* type A isolates, our current study constructed an isogenic *cpe* knock-out mutant of SM101, an electroporatable derivative of the *C. perfringens* type A foodpoisoning isolate NCTC 8798 which carries a chromosomal *cpe* gene, as well as F4969, a *Clostridium perfringens* type A nonfoodborne GI disease isolate which carries a plasmid-borne *cpe* gene. CPE-Western immunoblot analyses confirmed that both *cpe* knock-out mutants failed to express CPE and that this lack of CPE expression could be complemented by a recombinant plasmid carrying the wild type *cpe* gene. When the virulence of the wild-type, mutant, and complementing strains was compared in a rabbit ileal loop model, sporulating (but not vegetative) culture lysates of both wild-type isolates induced significant ileal loop fluid accumulation and intestinal histopathological damage. However, neither sporulating nor vegetative culture lysates of the *cpe* knock-out mutants induced these intestinal effects. Full sporulation-associated virulence could be restored by complementing either knock-out mutant with a recombinant plasmid carrying the wild-type *cpe* gene, which confirms that the observed loss of virulence for both *cpe* knock-out mutants results from the specific inactivation of the *cpe* gene and the resultant loss of CPE expression. These results now provide clear and direct evidence that CPE expression is necessary for both SM101 and F4969 to cause GI effects in the culture lysate:ileal loop model system, a finding which supports CPE playing an important role in the GI pathogenesis induced by SM101 and F4969 (and presumably by other *cpe*-positive *C. perfringens* type A isolates). These results also fail to implicate other toxins in the GI effects caused by SM101 and F4969.

THE ROLE OF PLC-INDUCED ACTIVATION OF PLATELET GPIIBIIA
IN CLOSTRIDIAL MYONECROSIS. AE Bryant, DL Stevens. Veterans
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Clostridium perfringens gas gangrene is a fulminant infection and radical amputation remains the single best treatment. We have hypothesized that rapid tissue destruction is related to tissue hypoxia secondary to toxin-induced vascular obstruction. Our previous studies demonstrated that phospholipase C (PLC) caused a rapid and irreversible decrease in skeletal muscle blood flow that paralleled the formation of intravascular aggregates of activated platelets, fibrin, and leukocytes. In this study, flow cytometry demonstrated that PLC stimulated platelet/neutrophil aggregation via activation of platelet gpIIb/IIIa. Pretreatment of animals with heparin or depletion of leukocytes reduced blood flow deficits and aggregate formation caused by PLC. We conclude that fulminant tissue destruction in gas gangrene results from profound attenuation of blood flow caused by PLC-induced, gpIIb/IIIa-mediated formation of heterotypic platelet/PMNL aggregates. Therapeutic strategies targeting gpIIb/IIIa may prevent vascular occlusion, maintain tissue viability, and provide an alternative to radical amputation for patients with this infection.

EFFECT OF INTRAVENOUS INJECTION OF *Clostridium perfringens*

TYPE D EPSILON TOXIN IN CALVES. F.A. Uzal¹, W.R. Kelly² and W.E. Morris¹.

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In cattle, a neurological lesion similar to that produced in sheep by *Clostridium perfringens* type D epsilon toxin has been reported. However, no causal relationship has been established between this toxin and the lesion in cattle. The effects of the intravenous injection of 120 MLD₅₀/kbw of epsilon toxin in 3 six month old calves were studied. Another calf was inoculated intravenously with saline solution and used as control. Epsilon toxin produced neurological signs within 110-133 minutes of intravenous injection in all the calves given this toxin. Clinical signs consisted of loss of consciousness, recumbency, convulsions, paddling, opistothonus, hyperesthesia and laboured breath. Gross changes consisted of severe acute pulmonary oedema that was particularly marked in the interlobular septae. The histological lesions in these animals consisted of intra-alveolar and interstitial oedema of the lung and variable degrees of perivascular proteinaceous oedema in internal capsule, thalamus and cerebellar peduncles. No clinical alterations or post-mortem changes were observed in the control calf. These results show that calves are susceptible to the intravenous injection of epsilon toxin, and that they can show at least some of the histological lesions produced in sheep and goats by this toxin.

PREVENTIVE EFFECT OF *CLOSTRIDIUM BUTYRICUM* ON
ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7
INFECTION

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Interaction between *C. butyricum* MIYAIRI 588 strain and enterohemorrhagic *E. coli* (EHEC) O157:H7 006 strain was examined in both streptomycin (SM)-treated SPF mice and germ-free mice.

All SPF mice pretreated with SM were colonized with EHEC O157:H7. On the other hand, only 25% of the SPF mice pretreated with SM and *C. butyricum* were colonized with EHEC O157:H7. The number (< 10³ cfu/g feces) of EHEC O157:H7 colonized in the mice treated with SM and *C. butyricum* was significantly less than that (10^{6.7} cfu/g feces) in the SM-treated mice. Interestingly, in the mice treated with SM and *C. butyricum* the number of non-O157 *E. coli* was recovered to 10⁷ cfu/g feces by 21 days after the infection.

All the gnotobiotic mice mono-associated with EHEC O157:H7 died with convulsion of extremities within 7 days after the infection. In contrast, all the gnotobiotic mice associated with *C. butyricum* survived after the challenge with EHEC O157:H7. The number (10^{7.8} cfu/g feces) of EHEC O157:H7 in the fecal contents of the gnotobiotic mice preinfected with *C. butyricum* was significantly less than that (more than 10⁹ cfu/g feces) in the gnotobiotic mice infected with only EHEC O157:H7. Similarly, the amounts of shiga toxin (ST1, ST2) in the fecal contents was significantly reduced in the gnotobiotic mice preinfected with *C. butyricum*.

In conclusion, the probiotic bacterium *C. butyricum* MIYAIRI 588, has a preventive effect on EHEC O157:H7 infection.

TXER, AN RNA POLYMERASE SIGMA FACTOR THAT ACTIVATES TRANSCRIPTION OF TOXIN GENES OF *CLOSTRIDIUM DIFFICILE*.

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The symptoms of disease caused by *C. difficile* vary from mild diarrhea to lethal pseudomembranous colitis. This variation seems to be due to differential expression of colonization and virulence factors, especially genes for production of toxins A and B. In strains that differ widely in toxin production and pathogenicity, the content of *tox* mRNA varied accordingly, suggesting that regulation of *tox* gene transcription is an important determinant of pathogenicity.

Although toxin gene expression is thought to involve TxeR, the product of the gene upstream of the *toxB-toxA* cluster, the mechanism of *tox* regulation was unknown. To determine the exact role of TxeR, C-terminal His tagged TxeR protein was partially purified by metal affinity chromatography and used in gel mobility shift assays. Our results show that neither TxeR alone nor core RNA polymerase is able to bind to the promoter regions of *tox* genes. When TxeR was mixed with core RNA polymerases of *E. coli* or *B. subtilis* or with *C. difficile* RNA polymerase, however, binding was observed. These results were consistent with *in vitro* run-off transcription assays and *in vivo* transcription assays in *C. perfringens*, using *ptox-gusA* fusions. Addition of TxeR to RNA polymerase from *C. difficile* or to core RNA polymerase of *E. coli* or *B. subtilis*, greatly stimulated *toxA*- and *toxB*-specific transcription.

These findings demonstrate that TxeR activates transcription of *tox* genes by acting as an alternative sigma factor. Interestingly, sequence analysis of TxeR shows significant homologies to the ECF class of sigma factors. ECF sigma factors generally respond to extracytoplasmic conditions; synthesis of TxeR is strongly influenced by nutrient availability.

REGULATION OF THE TOXINOGENESIS IN *CLOSTRIDIUM BOTULINUM* AND *CLOSTRIDIUM* *TETANI*

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Clostridium botulinum and *Clostridium tetani* produce potent neurotoxins. *Botulinum* neurotoxin (BoNT) is associated to non toxic proteins (ANTPs) to form high molecular weight complexes. In contrast, tetanus toxin (TeTx) does not form complex. The genes encoding the ANTPs are clustered in the vicinity of *bont* genes in all the *C. botulinum* toxinotypes. We have shown that in *C. tetani* the *tetx* gene is preceded by a gene encoding a basic 21 kDa protein (TetR) which is conserved (BotR) in *C. botulinum* strains. *botR* gene is localized at the 5' extremity of the *botulinum* locus in *C. botulinum* C and D, and between the two divergent operons encoding the BoNT and ANTPs in *C. botulinum* A, B and E. Overexpression of *botR/A* in *C. botulinum* A, and *tetR* in *C. tetani*, induces an overproduction of BoNT/A and TeTx respectively. Inversely, the repression of *botR/A* by a mRNA antisens method is accompanied by a decrease of BoNT/A production. *botR/A* regulates positively *bont/A* and also *antps* genes, whereas *tetR* seems to be only effective on *tetx* gene in *C. tetani*. *botR/A* and *botR/C*, transfected in *C. tetani*, increase the production of TeTx indicating that *C. botulinum* and *C. tetani* share a common regulation of the toxinogenesis. Specific mRNA assays show that BotR and TetR act at the transcriptional level. We have evidenced that BotR/A is able to bind the promoter regions of the two divergent operons encoding the *bont* and *antps* genes. Sequence analysis show that BotR and TetR seem to recognize a common DNA site.

REGULATION OF EXPRESSION OF TYPE A BOTULINAL NEUROTOXIN

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The molecular biology of botulinal neurotoxin gene expression in *Clostridium botulinum* type A is poorly understood. In this study, production of type A botulinal neurotoxin by *C. botulinum* strains Hall A, 62A, and NCTC 2916 was determined during 96 hours of growth. Strains were grown both in TPGY and in type A Toxin Production Media (TPM). Toxin concentrations in culture supernatants and lysed cell pellets were determined using ELISA and Western blots. For all three strains, regardless of the medium, levels of toxin released from cells peaked at the end of exponential growth. Toxin release into the culture supernatant varied, however, among strains and growth media. For *C. botulinum* strain Hall A, cell lysis and toxin release occurred rapidly for cells grown in TPM, while cells grown in TPGY remained in stationary phase with minimal lysis and toxin release through 96 hours of growth. For strain 62A, cell lysis and toxin release occurred to a greater extent in TPM compared to TPGY. For strain NCTC 2916, cell lysis and toxin release were not significantly different after 72 hours of growth in TPGY and TPM. Toxin production was highest in strain NCTC 2916, followed by Hall A and 62A. These data together with other findings in our laboratory indicate that toxin regulation in *C. botulinum* type A is a complex process involving genes affecting positive and negative promoter regulation, cell lysis, proteolytic activation of toxin, and lysis of cells with concomitant release of toxin into the media.

FUNCTIONAL ANALYSIS OF THE VirR RESPONSE REGULATOR FROM *Clostridium perfringens*

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The VirS/VirR two-component signal transduction system regulates the production of several extracellular toxins in *C. perfringens*, including *pfoA* which is the structural gene for perfringolysin O. We have purified a His-tagged VirR protein and shown that it binds to a 52-bp region located immediately upstream of the -35 box of the *pfoA* promoter. This region includes a directly repeated sequence (CCCAGTTNTNCAC) that is essential for VirR binding, as shown by site-directed mutagenesis and DNase I footprinting. Based on these data it is concluded that VirR activates *pfoA* by binding directly to the upstream promoter region. However, VirR does not bind specifically to other VirS/R regulated genes including *p/c*, *colA*, and *pfoR*, which suggests that the control of gene expression of these genes involves secondary regulators. We have also constructed and analysed two site-directed VirR mutants, D57N & K105E. These mutations are located in the conserved active site of the receiver domain of VirR; D57 is the proposed site of phosphorylation. Both mutant proteins were purified and shown to bind to the *pfoA* target site, which indicates that at least *in vitro* phosphorylation is not required for DNA binding. When these mutants were introduced into a *C. perfringens virR* mutant they were still able to complement the mutation, but not to wild-type levels. It is concluded that both the state of phosphorylation and the concentration of the VirR response regulator are important factors in the mechanism of action of this regulatory network.

A NOVEL RNA TRANSCRIPTIONAL REGULATOR THAT
CONTROLS TOXIN PRODUCTION IN *CLOSTRIDIUM*
PERFRINGENS

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Previously, we had identified novel VirR/VirS-regulated genes in *C. perfringens* (Mol. Microbiol. 2000 35:854-864). One of the genes named *hyp7* was shown to be responsible for the transcriptional activation of alpha-toxin (*plc*) and kappa-toxin (*colA*) genes. The *hyp7* gene was transcribed on a 0.4-kb mRNA (VR-RNA) whose expression was tightly regulated by the VirR/VirS system. Primer extension analysis revealed the VirR/VirS-regulated promoter located just upstream of the *hyp7* coding region. In the flanking region of the VR-RNA promoter, there found a DNA motif (CCAGTXXXXCAC) that resembled the repeated motifs found in the promoter region of the *pfoA* gene, indicating that the VirR protein could directly bind to this motif to activate the transcription of *pfoA* and VR-RNA. A deletion analysis of VR-RNA region revealed that only a 35-bp portion of the VR-RNA was enough for the regulation of the toxin genes. Furthermore, VR-RNA that was introduced a nonsense mutation in the *hyp7* coding region still activated the toxin genes. The secondary structure of VR-RNA had a strong axis of symmetry and many tight stem-loops. These results indicate that VR-RNA itself acts as a regulatory RNA for the production of alpha- and kappa-toxins and that the VirR/VirS system controls the expression of *plc* and *colA* via the VR-RNA regulatory system in *C. perfringens*.

THE LABORATORY DIAGNOSIS OF *CLOSTRIDIUM DIFFICILE* ASSOCIATED DIARRHOEA – IS IT TIME FOR A CHANGE?

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Clostridium difficile is the most common cause of hospital-acquired diarrhoea in the world. Patients who acquire *C. difficile*-associated diarrhoea (CDAD) remain in hospital, on average, an extra 15-20 days and each case costs approximately A\$12,500. Despite increases in our knowledge of the pathogenesis of CDAD, numbers of cases have continued to climb, primarily due to continued inappropriate use of cephalosporins and contamination of the hospital environment with *C. difficile* spores. Unfortunately, it is likely that for various reasons a significant amount of under-reporting is occurring. For diagnosis, many laboratories rely on only detection of toxin B in tissue culture or toxin A, or A and B, by immunoassay. This approach raises several important concerns. The detection of these toxins depends upon a number of factors, including their initial concentrations and lability, and variable excretion patterns. It is now also apparent that some *C. difficile* strains may lack a fully functional toxin A gene, and not produce an apparently biologically active toxin, and yet are still pathogenic. Finally, it is known that certain *C. difficile* clones are better than others in their ability to exist and spread in the hospital environment. However, until specific “epidemic” genes are described that might be detected with molecular techniques, to identify these clones requires that they are first cultured and then typed. Therefore, in order to overcome all these problems, it may be necessary to revisit procedures for the isolation of *C. difficile* in the diagnostic laboratory.

DIFFERENTIATION BETWEEN *CLOSTRIDIUM BOTULINUM* TYPE C/D AND *CLOSTRIDIUM NOVYI* TYPE A BY WESTERN BLOT OF SURFACE ANTIGENS - APPLICATION TO THE IDENTIFICATION OF VETERINARY ISOLATES FROM CASES OF DYSAUTONOMIA

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Clostridium botulinum type C and D and *Clostridium novyi* type A are indistinguishable by culture and biochemistry, and can only be separated by toxin detection. Toxigenicity is unstable due to loss of the phage encoding the toxin. This makes isolation of toxigenic organisms from specimens difficult, and subsequent identification of a non-toxigenic organism impossible.

The aims of this work were to develop a method to differentiate between *C. botulinum* type C and *C. novyi* type A and to apply this method to the identification of 16 isolates from the gut of animals with dysautonomia (9 equine, 1 feline, 1 leprine). These isolates had been identified as non-toxigenic *C. botulinum* type C or *C. novyi* type A. We have recently proposed that equine dysautonomia (equine grass sickness) is caused by a toxicoinfection with *C. botulinum* type C.

EDTA-extracted surface antigens of NCTC strains of *C. botulinum* types C/D and *C. novyi* type A were separated by SDS-PAGE and western blotted with antiserum to *C. novyi* type A surface antigens. This technique could differentiate between these organisms by a major band difference: 46kDa for *C. novyi* and 43kDa for *C. botulinum*. The 46kDa band was present in all the unknown isolates suggesting that they were *C. novyi* type A rather than non-toxigenic *C. botulinum* type C. Subsequently, detection of the alpha toxin gene by PCR in two of these isolates confirmed their identity as *C. novyi* type A. Similarities between isolates were observed despite the wide geographic range and type of host animals from which they were isolated.

C. novyi type A when cured of its phage can be transfected in vitro with the phage of *C. botulinum* type C or D and produce the neurotoxin. Can this happen in vivo, and if so is the difference between non-toxigenic *C. botulinum* type C and *C. novyi* type A clinically relevant?

A COMPARISON OF VACCINE DELIVERY MECHANISMS FOR A *CLOSTRIDIUM BOTULINUM* TYPE F TOXIN SUB-UNIT VACCINE.

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The neurotoxins produced by *Clostridium botulinum* are the most potent toxins known to man. Seven serotypes of the toxin exist (A to G) and exposure to microgram quantities of the toxins leads to a flaccid paralysis and death. The most likely sources of exposure to the toxin are through spoiled food and by accidental exposure of laboratory personnel working with the toxin. The current vaccine against the toxin, which consists of a pentavalent (A to E) toxoid mixture, has US Investigational New Drug status but is not licensed for human use. We are investigating the feasibility of producing sub-unit vaccines against the botulinum toxins. The sub-unit vaccine described here is based on the Hc (binding) domain of type F botulinum toxin (FHc). A synthetic gene encoding the FHc domain was expressed in *E. coli* as a maltose binding fusion (MBP-FHc). The cleavage product, FHc, was evaluated for its ability to protect against toxin challenge. Vaccination of Balb/c mice with the FHc by the intramuscular route resulted in protection against 10^4 LD₅₀ doses of type F botulinum toxin. In order to evaluate other methods of vaccine delivery, the MBP-FHc was microencapsulated and administered to mice via the intranasal and intramuscular routes. In a further study the feasibility of delivering the FHc in a live vaccine vector was investigated. The FHc construct was cloned into *S. typhimurium* was administered via the intravenous and oral routes. Protection against toxin challenge was achieved using both microencapsulation and a live vaccine vector as methods of vaccine delivery.

TUMOUR-SPECIFIC DELIVERY OF ANTI-CANCER AGENTS USING THE SPORES OF GENETICALLY MODIFIED CLOSTRIDIA

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Intravenously injected clostridial spores localise to, and exclusively germinate in, the hypoxic regions of solid tumours. They cannot grow in normal healthy tissue. The advances made in the last decade in clostridial genetics now provide the opportunity to capitalise on this unique specificity to deliver anti-cancer agents to tumours through appropriate genetic modification of the clostridial host. The agents that may be delivered fall into two classes (i) those substances which are anti-tumourogenic in their own right (eg., cytotoxins or cytokines), and (ii) prodrug-converting enzymes. Prodrug-converting enzymes are the central component of the so-called DEPT (directed enzyme prodrug therapy) strategies. In this approach, an enzyme is specifically targeted to a tumour where it brings about the conversion of an innocuous, circulatory prodrug into a highly cytotoxic species. This allows for the generation of relative high therapeutic doses of drug only within the vicinity of the tumour. In the first strategy devised, delivery was antibody-directed (ADEPT) through fusion of the enzyme to a tumour "specific" antigen. Thereafter, attention has focused on delivery of the gene (GDEPT) encoding the therapeutic enzyme, using virus-based or non-viral delivery vehicles. The analogous system using clostridial spores is termed CDEPT. Progress in this technology will be described.

Genetically engineered *Clostridia* expressing a prodrug activating enzyme for cancer gene therapy Liu, S.C., Shibata, T., Giaccia, A.J., Minton, N.P., and Brown, J.M. Dept. of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305-5468

Hypoxia is unique for human solid tumors and can be detrimental in cancer treatment. Non-pathogenic spore-forming *Clostridia* have been used experimentally as anti-cancer agents because of their selective germination and proliferation in hypoxic regions of solid tumors after systemic injection of their inactive spore form. We have proposed a novel approach for cancer gene therapy with genetically engineered *Clostridia* as a tumor-targeting vehicle. Using an electroporation technique, we have successfully transformed *C. sporogenes* (NCIMB 10696) with a plasmid encoding the prodrug-activating enzyme, an *E. coli* cytosine deaminase (CD). In an *in vitro* growth inhibition assay, incubation with lysates from transformed *Clostridia* increased the sensitivity of murine tumor cells to a non-toxic prodrug 5-fluorocytosine (5-FC) by more than 1,000 fold as compared with controls. In an *in vivo* experiment with transplantable tumors, significant anti-tumor effects could be achieved with 5-FC plus CD-producing *Clostridia*. The high level of prodrug activation, together with the high selectivity of *Clostridia* for hypoxic regions of tumors, indicates a great potential in cancer gene therapy.

THE LYSIS OF COMMERCIALLY AVAILABLE ANTI-CANCER LIPOSOMES BY *CLOSTRIDIUM PERFRINGENS* ALPHA-TOXIN

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LipoburstTM is a putative method of site directed anti-cancer therapy, involving localised release of chemotherapeutic drugs at tumour cell surfaces. An anti-tumour cell surface antibody conjugated to a phospholipase C (PLC) from *Clostridium perfringens*, alpha-toxin, provide the means for lysis of anti-cancer drug loaded liposomes at tumour sites. Previously reported murine *in vitro* studies showed the inhibition of cell proliferation for two human cell lines by LipoburstTM. In this study, we aimed to examine the enzymes kinetics behind the PLC-liposome interaction using two commercially available anti-cancer liposomes as model systems. The two liposomes investigated were daunorubicin loaded DaunoXomes® and PEGylated doxorubicin loaded CaelyxTM. Both enzyme and liposome concentration affected the rate of release of the entrapped drug. Further analysis of enzyme activity in the presence of the chemotherapeutic drug doxorubicin in standard PLC assays revealed that inhibition of activity occurred at high drug concentrations. PEGylation was also found to hinder the enzymes ability to lyse liposomes. The results generated will aid in the optimisation of the LipoburstTM system as a method of site directed anti-cancer therapy.

Poster Presentation

MAJORITY OF *CLOSTRIDIUM PERFRINGENS* STRAINS ISOLATED FROM FOOD ARE CPE-NEGATIVE

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Clostridium perfringens type A is an important cause of food borne infections in humans. Strains causing food poisonings have been found to harbor a *cpe*-gene, which encodes for enterotoxin production. The enterotoxin is produced during the sporulation of the bacteria in the small intestine. The aim of this study was to examine whether normal food samples harbour *cpe*-positive *C. perfringens* strains.

Fourty-seven *C. perfringens* strains isolated from variuos food in Germany were used in this study. The foods were not suspected samples from food poisoning cases. Two different sets of primers were used in the present study with two different PCR programs. The PCR products were run in 1% agarose gel with appropriate molecular size markers.

It was shown that only two strains of the 47 examined were *cpe*-positive. Both strains were positive with both sets of primers and all negative samples were negative in both experiments. This finding confirms that most of the *C. perfringens* strains isolated from normal food are *cpe*-negative. As these two positive strains were isolated from the same food sample, it should be examined whether they are clonal in further studies. Yet, another strain was isolated from this sample which was a *cpe*-negative. This suggests that several strains may exist in food, yet the ecology and diversity of *C. perfringens* in food, animals and environment requires further studies.

ISOLATION OF *CLOSTRIDIUM PERFRINGENS* FROM U.S.
RETAIL FOODS. Labbe, R. and Lin, Y.T. University of
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In recent years *C. perfringens* has been a leading cause of confirmed cases of human foodborne illness in the U.S. with temperature-abused meat and poultry foods as common vehicles. Approximately 130 food samples (meat, poultry, fish, dry seasonings) were obtained from various outlets in western Massachusetts. Forty confirmed isolates were obtained using the iron-milk medium for detection and lactose-gelatin and motility-nitrate as confirmatory media. Levels of *C. perfringens* were <100gm except in two cases, spare ribs (292/gm) and one sample of chicken leg (>1100/gm). No *cpe*-positive isolates were present as determined by gene probe tests or by the PCR. All isolates contained the *cpa* gene. Enterotoxin was not detected by reversed passive latex agglutination using cell extracts or culture supernatant fluids of sporulating cells of 22 isolates. Comparison of DNA restriction fragments by pulsed field gel electrophoresis identified no identical patterns among isolates in terms of number and sizes of fragments.

WHITEFISH ROE AS A VEHICLE FOR TYPE E BOTULISM IN FINLAND

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The first case of human botulism in Finland was diagnosed in November 1999 when a 54-year-old man got type E botulism from strongly spoiled home-prepared whitefish roe. Severe temperature abuse in the storage conditions of the fish roe was observed; after catching it had been inadvertently stored in a broken freezer with room temperature for two days. Thereafter the fish roe was lightly salted and stored for four days in the patient's refrigerator where the temperature was 11°C.

A total of two serum samples, four fecal and three gastric fluid samples from the patient as well as the suspected fish roe were investigated for the presence of botulinum toxin (BoNT) by the standard bioassay and for *C. botulinum* cells by BoNT-specific PCR. One gastric sample from the patient and the fish roe were shown to contain botulinum toxin. Toxin concentration in the fish roe was 6000 MLD/g. *C. botulinum* type E cells were detected from all fecal and gastric fluid samples as well as from the fish roe. Both serum samples were negative for botulinum toxin and cells. Pulsed-field gel electrophoresis (PFGE) analysis showed the *C. botulinum* isolates from the patient and the fish roe to be clonal.

A high prevalence of *C. botulinum* type E spores in the Baltic Sea and its fish has been reported which causes a high risk of botulism related to fish products. In the present case it is obvious that the reason for toxin production in the fish roe was the high storage temperature and a very low NaCl content (<1%).

GERMINATION-SPECIFIC SPORE CORTEX LYtic ENZYMES
FROM *C. PERFRINGENS* S40 SPORES: STRUCTURE AND
REGULATION OF ENZYMATIC ACTIVITY

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Bacterial spore germination is defined as a series of interrelated degradation events, triggered by specific germinants, which causes the irreversible loss of dormant spore characteristics. The process is controlled by sequential activation, or allosteric conformational alteration, of a set of germination-related enzymes preexisting in the dormant spore. One of the key enzymes is cortex hydrolases.

In *C. perfringens* S40 spores, at least two cortex-lytic enzymes are involved in cortex hydrolysis during germination; spore cortex-lytic enzyme (SCLE) which acts as an amidase and degrades intact cortex, and cortical fragment-lytic enzyme (CFLE) which functions as a muramidase and attacks disrupted cortex. We have examined time of synthesis, precursor structure and regulation of activity of these enzymes. Both enzymes are synthesized at the time of forespore formation during sporulation. SCLE is produced as a precursor consisting of an N-terminal presequence, an N-terminal prosequence, mature enzyme and an C-terminal prosequence. After the N-terminal presequence and the C-terminal prosequence are processed, the resulting inactive proenzyme is converted to active enzyme by cleavage of the N-terminal prosequence with germination-specific protease during germination. CFLE is produced as mature enzyme and does not need activation during germination. Expression of its activity is indicated to be regulated by the requirement of disrupted cortex as substrate. These results suggest that cortex hydrolysis during germination of this organism starts by cleavage of cortex cross-bridge with SCLE, which is followed by degradation of polysaccharide moiety of SCLE-modified cortex with CFLE.

VARIATION OF THE S-LAYER PROTEINS OF *CLOSTRIDIUM*

DIFFICILE

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Clostridium difficile expresses two major cell surface proteins, known as surface-layer (S-layer) proteins. The aim of the study was to investigate the variability of the S-layer proteins in *C. difficile*. S-layer proteins were extracted from 24 strains of *C. difficile*, each having been designated a Delmee serotype or a ribotype, by treatment with 5M guanidine hydrochloride. The extracts were run on 10% SDS-PAGE and visualised using Coomassie blue. Western blots using rabbit antisera were performed to determine the antigenic reactivity of the S-proteins. The guanidine hydrochloride extraction revealed two major proteins characteristic of the S-layer proteins and one minor protein common to all strains. Visual analysis of the two major proteins, showed high variability between strains. Molecular weight analysis using Phoretix software showed that the heavier of the two proteins varied between 47kDa and 62kDa, the smaller protein varied between 39kDa and 46kDa, the third minor, common protein was 70 kDa. Each isolate was designated a strain number based on the molecular weights of the two S-proteins using Phoretix software. At least 18 different strain numbers were demonstrated using this method. Further analysis by immunoblotting revealed the lighter protein to be immunogenic, but the heavier protein had no immunogenic reactivity. No cross-reaction was seen between any strains and immunoblotting could distinguish between strains that had the same or similar strain numbers. Our study shows a high degree of variability in molecular weight of the S-layer proteins from *C. difficile* and immunoreactivity appears to be strain specific. The S-layers may have a role in virulence and may be a useful marker for pheno-typing. Current analysis of several strains by protein sequencing analysis should further elucidate the mechanism and possible function of this S-layer variation.

ADAPTIVE RESPONSES TO OXIDATIVE STRESS OF *C. PERFRINGENS*

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C. perfringens, although referred to as a strict anaerobic bacterium, is widespread on the earth. In addition to humans and animals, it can be isolated from soil, water and air. In all these environments, the vegetative cells are repeatedly submitted to highly reactive oxygen species (ROS), mainly superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical, derived from the metabolism (reduction) of oxygen. Because the vegetative cells can survive these oxidative stress episodes, *C. perfringens* is likely to have developed efficient mechanisms of protection, which could also be important virulence factors.

The aerotolerance depends mainly on the environmental conditions. The sensitivity of *C. perfringens* to oxygen and its toxic derivatives was investigated in a new chemically defined synthetic medium, either in aerobiosis or anaerobiosis. The results obtained led to the conclusion that vegetative growing cells were more sensitive than stationary or glucose-starved cells. It was also shown that *C. perfringens* possessed an adaptive response to oxidative stress : pre-treatment of vegetative cells by short air exposure or sub-lethal concentrations of peroxides or redox-cycling agents, protected the cells against otherwise lethal challenge from these agents.

In order to identify the genes implicated in the stress response, mutants obtained after mutagenesis by the transposon Tn916, were screened for their lower or higher resistance to superoxide anion or hydroxyl/hydroperoxyl radicals, compared to the sensitivity of the wild type strain. Preliminary results of the genetic analysis of some of the mutants will be presented.

COMPARATIVE STUDIES OF THE EFFECTS OF HEATING ON
VEGETATIVE CELLS AND SPORES OF *CLOSTRIDIUM*
PERFRINGENS ISOLATES CARRYING PLASMID VERSUS
CHROMOSOMAL ENTEROTOXIN GENES.

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Clostridium perfringens enterotoxin (CPE) is an important virulence factor for both *C. perfringens* type A food poisoning and several nonfoodborne human gastrointestinal (GI) diseases. Recent studies indicate that *C. perfringens* isolates associated with food poisoning carry a chromosomal *cpe* gene, while nonfoodborne human GI disease isolates carry a plasmid *cpe* gene. However, no explanation has been provided for the strong associations between certain *cpe* genotypes and particular CPE-associated diseases. Since *C. perfringens* food poisoning usually involves cooked meat products, we hypothesized that chromosomal *cpe* isolates are so strongly associated with food poisoning because, i) they are more heat resistant than plasmid *cpe* isolates, ii) heating induces loss of the *cpe* plasmid, or iii) heating induces migration of the plasmid *cpe* gene onto the chromosome. When the current study tested these hypotheses, vegetative cells of chromosomal *cpe* isolates were found to exhibit, on average, ~2-fold higher D values at 55°C than the vegetative cells of plasmid *cpe* isolates. Furthermore, the spores of chromosomal *cpe* isolates had, on average, ~60-fold higher D values at 100°C than the spores of plasmid *cpe* isolates. Southern hybridization and CPE-Western blot analyses demonstrated that all survivors of heating retained their *cpe* gene in its original plasmid or chromosomal location, and could still express CPE. These results suggest that chromosomal *cpe* isolates are strongly associated with food poisoning, at least in part, because their cells and spores possess a high degree of heat resistance, which should enhance their survival in incompletely cooked or inadequately warmed foods.

VIRULENCE FACTOR DISCOVERY IN *CLOSTRIDIUM*
DIFFICILE THROUGH MODULATION OF
TRANSCRIPTION FACTOR EXPRESSION USING ANTI-
SENSE RNA.

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The problems associated with *C. difficile*-associated disease management represent a significant burden on the resources of National Health services. This burden will increase with an ageing population. The rational development of therapeutic strategies to counter the threat of bacterial pathogens requires a detailed knowledge of the molecular basis of virulence. The pathogenesis of *C. difficile* infection is, however, poorly understood. Thus, the two large toxins elaborated by this organism, toxin A and toxin B, represent the only known virulence factors. We have, therefore, devised a strategy which should enable the identification of virulence factors using anti-sense technology. A replication-deficient recombinant expression vector (pMTL940C) has been constructed into which has been inserted a DNA fragment specifying an anti-sense RNA molecule directed against the *C. difficile* homologue of the *C. perfringens* *virR* gene. This plasmid maybe delivered to the *C. difficile* genome, from a *Bacillus subtilis* donor, following its cointegration with Tn916. Inhibition of VirR production should lead to the down-regulation of those genes under its control, the products of which may be visualised on 2-D gels.

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PRELIMINARY SEQUENCE ANALYSIS OF RNA POLYMERASE GENES OF *CLOSTRIDIUM DIFFICILE*

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Clostridium difficile, an anaerobic endospore-forming bacterium, is a major nosocomial pathogen. It is associated with 10-25% of cases of antibiotic associated diarrhea (AAD) and almost all cases of pseudomembranous colitis (PMC). To gain a better understanding of sporulation of *C. difficile*, it was our goal to identify homologs of genes known to regulate this developmental process in *Bacillus subtilis*, i.e. the sporulation-specific sigma factors of RNA polymerase.

In our analysis we identified five sporulation-specific sigma factor genes, as well as eight additional sigma factor genes in the *C. difficile* genome. Genes were identified through heterologous PCR amplification techniques and by analysis of the increasingly available genome sequence. The identified sigma factors show striking homology to the *B. subtilis* proteins and are nearly identical in regions known to interact with promoter DNA sequences. This high level of sequence similarity has led us to predict that *C. difficile* utilizes promoters similar to those of *B. subtilis*. We have also shown that the organization of operons encoding the sporulation-specific sigma factors of *C. difficile* is the same as that in *B. subtilis* and *C. acetobutylicum*. We were surprised to find, however, that in strain CD630 the gene for the sporulation-specific σ^K appears to be disrupted by a prophage-like insertion. Until now *B. subtilis* has been the only spore-former shown to carry such an insertion. Other *Bacillus* and *Clostridium* species contain intact *sigK* genes. This finding raises interesting questions about evolution and selective advantages.

Demonstration that the Site-Specific Recombinase TndX Encoded by the Conjugative Transposon Tn5397 from *Clostridium difficile* is Required and Sufficient for Integration and Excision in *Escherichia coli*

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Tn5397 is a 21 kb tetracycline resistance encoding conjugative transposon isolated from *Clostridium difficile*. This element can transfer between *C. difficile* strains and to and from *Bacillus subtilis*. It encodes a conjugation system which is very similar to that of Tn916 and a completely different insertion/excision system. To investigate transposition of this element, two mini-transposons derived from Tn5397 were constructed. One represents the linear parental transposon and the other represents the circular form. These were used in excision and insertion assays respectively. We showed that the mini-transposons can be excised from and inserted into the original target site from *C. difficile* or from *B. subtilis* when that is present on a vector plasmid in *Escherichia coli*, upon expression of the element encoded site-specific recombinase, TndX. TndX belongs to the newly described large resolvase subgroup of the resolvase/invertase family of site-specific recombinases. Analysis of the target sites showed the cloned Tn5397 targets from *C. difficile* and *B. subtilis* were still hot spots for the mini-transposon insertion in *E. coli*.

Demonstration of Splicing of the Group II Intron present in the clostridial Conjugative Transposon Tn5397 and its Role in Conjugation.

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Tn5397 is a tetracycline resistance encoding conjugative transposon isolated from *Clostridium difficile* strain 630. This conjugative transposon can transfer between *C. difficile* strains and to and from *C. difficile* to *Bacillus subtilis*. Previous studies have shown that the element is related to the well studied conjugative transposon Tn916 over much of its length but there are some important differences. The ends of the two elements, which contain the integration and excision systems, are completely different. Furthermore, Tn5397 contains a group II intron. We have investigated the splicing of this intron from the RNA of both *C. difficile* and *B. subtilis* using RT-PCR. The results showed that both spliced mRNA and unspliced pre-mRNA were present in both species at all growth phases investigated. An intron mutant in which a kanamycin resistance gene replaced part of the intron *orf* was constructed. This mutant intron failed to splice. The effects of the mutation on the excision and conjugative transfer of Tn5397 are reported.

CHARACTERISATION OF THE SPECIFICITY OF
THE RESTRICTION/METHYLATION SYSTEM OF
CLOSTRIDIUM DIFFICILE AND THE
DEVELOPMENT OF SHUTTLE VECTORS.

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Whilst *Clostridium difficile* produces two potent toxin molecules, other factors undoubtedly contribute to virulence. Ignorance of pathogenesis in this organism is exacerbated by the lack of effective gene systems to facilitate its analysis. Thus, there is currently no described procedure for transforming this important human pathogen. To develop such a system we have elected to generate a vector based on an endogenous *C. difficile* plasmid, and to rationally counter the effects of the organism's restriction systems through appropriate methylation of the plasmids employed. Accordingly, a plasmid has been isolated from *C. difficile* and its replication functions identified following the determination of its entire nucleotide sequence. Shuttle vectors based on this replicon have been shown to be capable of transforming, and to be stably maintained in, *Clostridium beijerinckii*. In parallel, we have determined that *C. difficile* produces four type II restriction/modification systems. The restriction/methylation specificity of two of these has been determined and their effects countered through the production of plasmid vectors in an appropriate bacterial host. Characterisation of the other two is ongoing.

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COMPLETE NUCLEOTIDE SEQUENCE OF 54-KB
VIRULENCE PLASMID OF *CLOSTRIDIUM PERFRINGENS*
STRAIN 13

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In the course of the whole genome sequencing on *C. perfringens* strain 13, we found that strain 13 has a 50-100 kb plasmid that was not digested by *Nru*I, *Mlu*I, *Apal*, and *Sfi*I. By pulsed field gel electrophoresis (PFGE) and Southern blotting, this plasmid was shown to carry a gene encoding beta2 toxin (*cpb2*) that was reported to be one of the virulence factors of *C. perfringens*, indicating that the plasmid might play an important role for the pathogenicity of this strain. During the contig assembly, we found that contig180 contained a nucleotide sequence of the *cpb2* gene. To test that contig180 consists of a circular plasmid, a PCR was performed against total DNA from strain 13 using primers that annealed to both ends of contig180. The PCR was successful and the gap between both ends of contig180 was filled by a direct sequencing. The virulence plasmid was 54,702 bp in length and named pCP13. On pCP13, there found many open reading frames including resolvase, transposase, and transfer-related genes. We found that a gene similar to collagen adhesin gene (*cna*) of *Staphylococcus aureus* was located on pCP13, indicating that the *cna* gene might be important for attachment of *C. perfringens* to collagens of human tissue.

GENOME STRUCTURE OF A BOTULINUM TOXIN TYPE C-
CONVERTING PHAGE, c-st

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The gene structure of the c-st phage which is a botulinum type C toxin converting phage isolated from a C-Stockholm (C-ST) strain. In this study, we analyzed the structure of the c-st phage genome by pulsed-field gel electrophoresis (PFGE). The c-st phage was propagated on an indicator strain, (C)-AO2. After treating the phage lysate with RNase A, phage particles were recovered by polyethylene glycol precipitation and resuspended in TE buffer. Phage particles were embedded in agarose plug, treated with proteinase K, and subjected to PFGE analysis. The intact phage DNA preparation exhibited a single band of 160 kb. The phage DNA was digested at a single site by *Nru* I, *Mlu* I, *Mro* I, *Nhe* I, *Stu* I, and *Bsi*W I, respectively, while at two sites by *Kpn* I, at three sites by *Pvu* II, and at four sites by *Sal* I. The size of the phage genome deduced from the restriction enzyme cleavage map constucted was approximately 160 kb, which was in a good agreement with the value obtained from the analysis of the intact phage DNA. However, DNA fragments derived from both ends of the phage genome exhibited a ladder pattern, suggesting that the phage genome might be a circularly permuted and terminally redundant DNA. To gain more insights into the structural feature of the phage genome and the molecular mechanism underlying the acquisition of the botulinum toxin gene by the bacteriophage, determination of the whole genome sequence of the c-st phage is now under progress

ANALYSIS OF TEN RIBOSOMAL RNA OPERONS OF *CLOSTRIDIUM PERFRINGENS* STRAIN13

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The *Clostridium perfringens* genome comprises ten copies of the rRNA operon. The fact that the *E. coli* have seven copies of the rRNA operon per genome and *Mycobacterium tuberculosis* have only one, suggests that the copy number of rRNA operon is correlated with the rate of bacterial growth. In this way, since there was relationship between the mode of bacterial growth, and the copy number and the location of rRNA operon, we cloned and sequenced all ten rRNA operons from *Clostridium perfringens* strain13. As a result, the sizes of rRNA operons were 4.7-4.9 kb and each of those has a strikingly high homology. All ten rRNA operons situated on the genome map of the *C. perfringens* strain13. Those ten clustered in a region representing about one-third of the genome around the origin of replication and seven were transcribed clockwise and the remaining three were transcribed in a counter-clockwise direction. Two of the ten rRNA operons contained tRNA-Ile genes between the 16S and 23S rRNA genes. This situation is similar to that of other *C. perfringens* and *Bacillus subtilis*. One was substituted tRNA-Asn gene for the 5S rRNA gene.

MOLECULAR COMPOSITION OF PROGENITOR TOXIN PRODUCED BY *CLOSTRIDIUM BOTULINUM* TYPE C STRAINS 6813 AND 6814

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The molecular compositions of the purified progenitor toxins (M and L) produced by *Clostridium botulinum* type C strains 6813 (C-6813) and 6814 (C-6814) were analyzed. Furthermore, the entire nucleotide sequences of the genes coding for the progenitor toxin were determined. The dual cleavage sites at disulfied loop region of the dichain structure neurotoxin (NT) were identified by the analysis of C-terminal and N-terminal amino acid sequence of L and H chains. Analysis of partial amino acid sequences of fragments generated by limited proteolysis of NT showed that the NTs produced by both strains were hybrid molecule composed of type C and D NT as previously reported. Hemagglutinin (HA) components consist of mixture of several subcomponents with molecular mass of 70-, 55-, 33-, 26~21-, and 17-kDa proteins. The N-terminal amino acid sequences of HA subcomponents indicated that 70-kDa protein was intact HA-70 gene product, and other 55-, 26~21-kDa proteins with several different N-terminus were derived from the 70-kDa protein. The identity of deduced amino acid sequences of the progenitor toxin components of C-6813 and C-6814 were 99%, indicating that both strains were closely related. Furthermore, 92~97% identities among type C and D strains were exhibited in the deduced amino acid sequences of the genes for the nontoxic-nonhemagglutinin (NTNHA) and HAs which were considered to be common among type C (C-St and C-468) and D (D-CB16 and D-1873) progenitor toxins.

DIVERSITY OF NUCLEOTIDE SEQUENCE OF *CLOSTRIDIUM PERFRINGENS* EPSILON-TOXIN GENE

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Among the four major lethal toxin genes of *Clostridium perfringens*, alpha-toxin gene is encoded on chromosome, however, beta-, epsilon- and iota-toxin genes are located on plasmids. In addition, It has been reported that the nucleotide sequence of epsilon-toxin gene of *C. perfringens* type B was different in part from that of type D.

We encountered a phenomenon that, for a long-term preservation in cooked meat broth, four *C. perfringens* type B strains lost their beta-toxin gene and consequently these strains were identified as type D. The regions including promoter and ORF of epsilon-toxin gene of the four reassessed type D strains were amplified by PCR method and were sequenced in comparison with those of five type D strains. Two among the four reassessed type D strains possessed the identical sequence with those of type B strains (epsilon B), while the other two possessed a different sequence, which was identical to those of three out of five type D strains (epsilon D₁). The other two among five type D strains showed the identical sequence with that of type D with the exception of only one nucleotide change in ORF (epsilon D₂).

ROLE OF THE N- AND THE C-DOMAINS OF *CLOSTRIDIUM PERFRINGENS* ALPHA-TOXIN ON BIOLOGICAL ACTIVITIES

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The C-domain of alpha-toxin has been reported to be important for biological activities such as hemolytic and phospholipase C (PLC) activities, especially a calcium binding site. Despite these previous studies, understanding of the relationship between biological and enzymatic activities and the C-domain of the toxin remains incomplete. To characterize the C-domain of the toxin, fusion proteins consisting of the N- and C-domains of alpha-toxin and *C. bifermentans* phospholipase C (CbPLC), and *Bacillus cereus* phospholipase C (BcPLC) were constructed. The N-domain (1-250 residues; Ncp) of alpha-toxin showed about 4 % of PLC activity of wild-type alpha-toxin (WT), but no hemolytic activity. PLC activity of N-domain of alpha-toxin-C-domain of CbPLC (NcpCcb) was lower than that of WT, and higher than that of Ncp (10 % activity of WT). Hemolytic activity of NcpCcb toward sheep and rabbit erythrocytes was about 0.5 and 30 % of the WT activity, respectively. CbPLC was about 10 % of PLC activity of WT, but showed little hemolysis and 0.2 % hemolysis toward sheep and rabbit erythrocytes, respectively, compared with those of WT. Hemolytic and PLC activities of WT and N-domain of CbPLC-C-domain of alpha-toxin (NcbCcp) were similar. BcPLC-C-domain of alpha-toxin (NbCccp) significantly hemolyzed rabbit erythrocytes, but did not sheep erythrocytes. BcPLC and BcPLC-C-domain of CbPLC (NbCccb) showed no hemolysis. PLC activity of NbCccp and NbCccb was not significant from that of BcPLC. WT, NbCccp and NbCcp strongly bound to rabbit erythrocytes, CbPLC and NbCccb faintly did, and Ncp and BcPLC did not. When the C-domain of alpha-toxin was added to Ncp and BcPLC, hemolytic activity was completely restored and significantly observed, respectively. These observations showed that the C-terminal domain of alpha-toxin plays an important role in binding to substrates and/or erythrocytes of the N-domain.

PREVENTION OF ABSORPTION OF THE PROGENITOR TOXINS
FROM THE SMALL INTESTINE BY LOCAL IMMUNITY
AGAINST THE NONTOXIC COMPONENTS ASSOCIATED WITH
CLOSTRIDIUM BOTULINUM NEUROTOXINS

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C. botulinum types C and D neurotoxins are associated with nontoxic components (designated non-toxic non-HA and HA) and become large complexes (designated progenitor toxin) with 300 and 500 kDa in their cultures. It is reported that the nontoxic components are necessary to cause food-borne botulism because they protect the neurotoxins from gauntlet of gastric juice. We demonstrated that hemagglutinin (HA) of type C toxin binds to the epithelial cells of the small intestine, and leads to effective absorption of the 500 kDa toxin from the small intestine in guinea pig. Since the amino acid sequences of C and D nontoxic components are almost identical, it was presumed that anti-HA antibody produced in the small intestine might prevent the absorption of both C and D toxins from the small intestine.

We previously prepared a mutant, (C)-N71, which produces no neurotoxin but whole nontoxic components, and a mutant of *E. coli* LT toxin that has low toxicity but possesses high mucosal adjuvant activity. The nontoxic components purified from (C)-N71 were mixed with the LT mutant and then immunized the mice through nasal route four times at 1-week intervals. When these mice were orally challenged with either C or D 500 kDa toxin or their mixtures, showing 4 times of minimum oral lethal doses (about 4×10^4 mouse i.p. LD₅₀) no mice died, and high levels of Ig A and Ig G against the nontoxic components were detected in the washing fluids of their small intestine. These indicate that antibody against type C HA prevents the absorption of both type C and D HA-positive 500 kDa toxins from the small intestine.

CHARACTERIZATION OF HEMAGGLUTININ ACTIVITY OF
C. BOTULINUM TYPE A HA POSITIVE PROGENITOR TOXIN
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C. botulinum neurotoxin (~150 kDa) associate with nontoxic components in the culture fluids to form larger complexes designated progenitor toxins. In the type A strain, three different sized progenitor toxins with molecular masses of 900 kDa (19S), 500 kDa (16S), and 300 kDa (12S) are produced. The 12S toxin is formed by association of a neurotoxin with a nontoxic component having no hemagglutinin (HA) activity, while 19S and 16S toxins are formed by conjugation of the 12S toxin with a nontoxic component having HA activity. We previously purified type A progenitor toxins and analyzed the molecular composition of these toxins.

In this study, HA activity of *C. botulinum* type A 19S and 16S toxins (HA positive progenitor toxin) was characterized. Type A HA positive progenitor toxin agglutinated human erythrocytes. HA titers were decreased by the addition of lactose, D-galactose, N-acetyl-D-galactosamine, and D-fucose to the reaction mixtures. A direct glycolipids-binding test demonstrated that type A HA positive progenitor toxin strongly bound to paragloboside, but did not bind to gangliosides. A direct glycoproteins-binding test demonstrated that the toxin bound to asialofetuin, but not to fetuin. When the asialofetuin was first treated with endo- α -N-acetylgalactosaminidase, the binding of type A HA positive progenitor toxin was not affected. However, the toxin did not bind to glycopeptidase F treated-asialofetuin. Therefore, it is concluded that type A HA positive progenitor toxin recognizes and binds to Gal β 1-4GlcNAc contained in paragloboside and the N-linked oligosaccharides of glycoproteins.

***Clostridium perfringens* Iota toxin: Mapping of Receptor and Ia docking Domains of Ib.**

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Iota toxin is a binary enterotoxin consisting of iota a (Ia), which possesses ADP-ribosylating activity, and iota b (Ib) which binds to a cell surface receptor and translocates Ia into the cell. A set of Ib deletion mutants were tested for binding to Vero cells and docking with Ia via flow cytometry. Additional from competition of peptides with iota domain cytotoxicity assays revealed that the C-terminus of Ib binds a cell surface receptor, while the N-terminus interacts with Ia.

Five monoclonal antibodies towards Ib were tested with the deletion mutants were used for epitope mapping with the surface plasmon resonance technology. Three antibodies recognized the first 66 amino acids of the N-terminus, and two recognize epitope(s) within the 200 C-terminal residues.

Substrate recognition of the Collagen-binding Domain of the *Clostridium histolyticum* Class I Collagenase

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Clostridium histolyticum produces two distinct collagenases. We have identified the gene encoding class II collagenase (ColH), and revealed its segmental structure (S1+S2a+S2b+S3). When a C-terminal fragment, S2b+S3 was fused to the basic fibroblast growth factor (bFGF), it remained in the type I collagen-rich tissue to exert a growth-promoting effect much longer than bFGF *per se*. Thus, the fragment can be utilized as an anchoring unit in a drug delivery system which makes therapeutic agents non-diffusible and long-standing *in vivo*. In order to replace the anchoring unit with low-molecular mimics, it is essential to understand the underlying principles of the interaction.

Recently we have cloned the gene encoding class I collagenase (ColG) possessing a different structure, S1+S2+S3a+S3b. S3 from this enzyme bound to various types of collagen as monomeric (S3b) and tandem (S3a+S3b) form. They bound to atelocollagen lacking telopeptides, but not to the immobilized telopeptides. These observations suggested that the binding site(s) is present in the collagenous region. S3 bound well to collagen-like peptides, (Pro-Hyp-Gly)_n, only when n is large enough to allow the peptide to have triple helical conformation. S3 behaved in the same manner against another collagen-like peptides, (Pro-Pro-Gly)_n, but failed to bind to the similar peptides or gelatin lacking proper triple helical conformation. It was concluded that S3 contains the collagen-binding domain which recognizes the triple helical conformation of collagenous region.

REGULATORS OF GENE EXPRESSION DURING STATIONARY PHASE
IN *CLOSTRIDIUM PERFRINGENS*.

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Clostridium perfringens is a ubiquitous pathogenic bacterium in humans and domestic livestock. For its success production and secretion of toxins and proteases is essential. In order to identify clostridial regulators of toxin and protease production we constructed a chromosomal DNA library of *C. perfringens* NCTC 8257 (Ian R. Poxton, University of Edinburgh, UK) into the multicopy vector pHT315 (1). To search for putative regulators the clostridial DNA library was introduced by transformation of competent cells into a derivative of the reference wild type strain JH642 of *Bacillus subtilis*. This strain harbors a transcriptional lac fusion to the alkaline protease promoter (*apr-lacZ*, James A Hoch, The Scripps Research Institute, La Jolla, CA, USA). The working hypothesis was that general mechanisms controlling gene expression, especially during stationary phase, would be common to all species of endospore forming bacteria like the case of *Clostridium* and *Bacillus*. In consequence, constitutive over-expression of clostridial regulators will affect *apr* expression in *Bacillus* in a similar way than into clostridia. After an initial screening of 4,000 *Bacillus* transformants we identified four and two clones harboring clostridial fragments that decreased and increased *apr* expression respectively. Also, one of the clostridial inhibitor clones produced a dramatic decrease on the efficiency of spore formation when evaluated for cell survival after heat or chloroform treatment. The clostridial inserts were cloned and sequenced and their nature and a rational pictured of their function will be presented.

(1) Wang L, Grau R, Perego M, and Hoch JA. Genes & Dev 11(19) 97: 2569-2579.

BINDING OF THE RNA POLYMERASE α SUBUNIT TO PROMOTER UPSTREAM BENT DNA OF THE *Clostridium perfringens* PHOSPHOLIPASE C GENE

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The *Clostridium perfringens* phospholipase C gene (*plc*) possesses three phased A-tracts upstream of the promoter (-66 to -40). The A-tracts form bent DNA, facilitate the formation of the RNA polymerase (RNAP)-*plc* promoter complex through extension of the contact region, and activate the transcription in a low temperature-dependent manner. In order to clarify the mechanism of the transcriptional regulation by the A-tracts, it seems important to elucidate specific subunit/domain of RNAP and the A-tracts responsible for the extended contact.

We cloned *rpoA* encoding α subunit of the *C. perfringens* RNAP. The deduced amino acid sequence was identical to the N-terminal sequence of purified RNAP α subunit. The coding region of *rpoA* was cloned into an expression vector to overproduce and purify the α subunit (α -WT, 315 aa). N-terminal (α -NTD, 228 aa) and C-terminal (α -CTD, 79 aa) domains of the subunit were also purified in the same manner.

The gel retardation assays showed that α -WT and α -CTD bind to a DNA fragment containing three phased A-tracts and *plc* promoter (3Ap), but that α -NTD does not. From this result, a possibility was raised that α -CTD binds to the A-tracts. We are trying to show the contact region between 3Ap and α -CTD by hydroxyl radical footprinting at the moment.

REGULATION OF *CLOSTRIDIUM DIFFICILE* TOXIN GENES
BY TxeR

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The regulation of *toxA* and *toxB* genes of *Clostridium difficile* by TxeR was studied using *in vitro* and *in vivo* transcription systems. *C. difficile* RNA polymerase was purified using differential ammonium sulfate precipitation, anion exchange chromatography on DEAE-sepharose columns and cation exchange chromatography on phosphocellulose columns. In an *in vitro* run-off transcription assay, addition of TxeR to *C. difficile* RNA polymerase greatly stimulated *toxA* and *toxB* specific transcription. TxeR also conferred *toxA* and *toxB* promoter recognition to both *E. coli* and *B. subtilis* RNA polymerases. Sequence analysis of TxeR suggests significant similarities to the ECF class of sigma factors. Interestingly, TxeR was able to stimulate transcription from the *tox* promoters with *E. coli* RNA polymerase core enzyme, thereby suggesting that TxeR could be an alternative sigma factor regulating toxin gene expression in *C. difficile*. When expressed *in trans* in *Clostridium perfringens*, *txeR* not only activated a *toxA-gusA* and a *toxB-gusA* fusion, but it also stimulated a *txeR-gusA* fusion, suggesting a positive autoregulatory loop. Based on these results and those of others we propose a tentative model for *tox* gene regulation as follows: As *C. difficile* cells reach stationary phase, a signal (such as nutrient depletion, stress, etc) results in the synthesis of TxeR. TxeR then positively activates its own transcription and transcription from *tox* promoters by acting as an alternative sigma factor. In the presence of a rapidly metabolizable carbon source, such as glucose, the signal that leads to the synthesis of TxeR is suppressed and hence toxin synthesis is greatly reduced.

VIRULENCE ACTIVATING PHEROMONE (VAP) OF
CLOSTRIDIUM PERFRINGENS

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One mutant strain of *C. perfringens* strain 13, SI112 that scarcely produce alpha-, kappa-, and theta-toxins was isolated. When the strain SI112 was cross-streaked with the *virR/virS*-mutant strain TS133, production of theta-toxin from SI112 was clearly observed in the crossing portion on a sheep blood agar plate. This indicated that an extracellular stimulating substance released from TS133 activated the production of theta-toxin from SI112 that could not produce the substance. We tentatively named the substance as VAP (virulence activating pheromone) and analyzed further. VAP in conditioned medium (CM) was produced mainly during the exponential phase and activated theta-toxin production at the transcriptional level. The stimulation of the *pfoA* gene by CM appeared to be dose-dependent. CM activated the *pfoA* transcription mostly at 15 min after addition, indicating a quick response to VAP exists in *C. perfringens*. VAP in CM seemed to be highly unstable loosing its activity within 30 min at 37°C, and appeared to be a small molecule (M.W.<10,000). Since this extracellular communication for the virulence of *C. perfringens* would be important for understanding the pathogenicity and preventing infections, we are now trying to purify VAP from the culture medium of strain 13.

IDENTIFICATION OF A NOVEL LOCUS THAT
CONTROLS TOXIN PRODUCTION IN *CLOSTRIDIUM*
PERFRINGENS

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Extracellular toxin production in *Clostridium perfringens* is positively regulated by a two-component regulatory genes, *virR* and *virS*. Comparative analysis of VirR/VirS-dependent promoters of toxin genes did not reveal any sequence motifs common to the promoter regions of VirR/VirS-regulated toxin genes. Therefore, VirR/VirS system may regulate the expression of toxins using secondary regulators in *C. perfringens*. To identify the putative secondary regulators for toxin genes, a chromosomal DNA library constructed from strain 13 was transformed into a mutant strain SI112 that lacks toxin production. One strain out of 3,000 transformants restored production of alpha-, theta-, and kappa-toxins, and the transformant contained a 3.2-kb DNA fragment on the plasmid. Deletion analysis showed that only 0.4-kb region was sufficient for the activity to restore toxin production in SI112. We constructed a mutant strain of this region by an allelic exchange method, and the resulting mutant showed much weaker transcription of the *plc*, *pfoA*, and *colA* genes. These results indicate that the 0.4-kb region plays an important role in the regulation of the toxin genes.

TETANUS IMMUNIZATION: CURRENT SITUATION IN JAPAN

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Since the introduction of tetanus vaccination in Japan 50 years ago, a remarkable decrease in incidence of this disease has been recorded.

After reaching to less than 50 cases in 1985, the number of the annual cases has been constant. Advances in technology for treatment of patients has also contributed to a continuous decrease of mortality in recent years.

The recommended schedule for routine tetanus immunization includes 4 DTaP doses between 3 and 90 months of age and 1 DT dose at the age of 11 to 12 years, and no additional vaccination for adults is scheduled. In order to evaluate the effectiveness of the schedule, we measured anti-tetanus titers of 881 serum samples collected from

Japanese people ranging 0 to 100 years, using Tetanus Antibody Assay Kit (Kaketsuken Ltd, Kumamoto, Japan).

About 85 % of Japanese people below 27 years possessed anti-tetanus titers higher than the preventive level (0.01 U/ml), indicating the effectiveness of the schedule to this generation. However, obvious decrease in titer was observed with increasing age, about 90 % of people over 27 years exhibiting non-preventive level (< 0.01 U/ml). These results are consistent with the fact that tetanus occurs almost exclusively among non- or inadequately vaccinated persons, in aged-population and suggests the importance of booster immunization for adults, to keep the preventive level against tetanus.

Development and evaluation of 16S rRNA-gene-targeted group-specific primers and probe for *Clostridium* cluster XIV in the human intestine.

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Clostridia are one of the main components of the normal diversified and complex microbiota of the human intestine. Clostridia are defined as spore-forming, obligatory anaerobic Gram-positive bacilli. Nonetheless, according to the 16S rRNA classification system, Clostridia are classified as belonging to the low-G+C-content Gram-positive group, consisting of 19 clusters(Collins et. al. 1994. Int. J. Syst. Bacteriol. 44:812-826). The results of recent studies in the field of molecular biology have shown that most strains of predominant Clostridia of the normal human intestinal flora belong to cluster XIV. Therefore, based on the 16S rRNA sequences retrieved from the DDBJ database, cluster-specific primers for the *Clostridium* cluster XIV were prepared, and their specificity was investigated using 14 strains of the *Clostridium* cluster XIV and 39 strains of other typical intestinal flora components. By utilizing DNA fragments extracted from 45 adult human fecal samples as templates, PCR was performed using these primers. The results confirmed the target PCR products in every sample. Furthermore, after incubating five adult human fecal samples using non-selective culture media used for total bacterial cell counting, isolated bacterial strains were identified using these primers. The results showed that the *Clostridium* cluster XIV accounted for 24±6% of the cultivable bacterial strains. Also, the results of a fluorescent in-situ hybridization (FISH) analysis using the cluster-specific probe showed that the *Clostridium* cluster XIV accounted for 14±2% of the total bacterial strains, thus suggesting that the *Clostridium* cluster XIV is found widely and predominantly in the adult human intestine.

PCR DETECTION OF *Clostridium chauvoei*. F.A. Uzal¹, P. Hugenholtz², S. Petray¹, L.L. Blackall², S. Moss³, R.A. Assis⁴, and W.E. Morris¹. 1-Animal Health Unit, The National Institute of Agricultural Technology, CC 277 (8400) Bariloche, Argentina. E-mail: fuzal@bariloche.inta.gov.ar, 2- Department of Microbiology and Parasitology, and 3-School of Veterinary Sciences and Animal production, The University of Queensland, Brisbane, Australia. 4-Veterinary School, Federal University of Minas Gerais, Belo Horizonte, Brazil.

A polymerase chain reaction (PCR) was used to amplify specific segments of the 16S ribosomal RNA gene of *Clostridium chauvoei*, a major pathogen of ruminants. Five sets of primers were designed to produce amplicons of 159, 836, 837, 959 and 960 basepairs (bp), respectively. The PCR was evaluated by testing 20 different strains of *C. chauvoei*, 7 strains each of *Clostridium septicum* and *Clostridium perfringens* and 2 strains each of *Clostridium novyi*, *Clostridium histolyticum* and *Clostridium sordellii*. Both purified DNA and biomass from pure cultures of each of these microorganisms were evaluated as templates in the PCR. In addition, extracts of formalin-fixed, paraffin-embedded tissues of 2 sheep and 2 guinea pigs experimentally inoculated with *C. chauvoei* were also tested by the PCR using the five sets of primers. Purified DNA template of all *C. chauvoei* strains produced PCR amplicons of the expected size for all five primer pairs. However, when biomass from pure cultures of *C. chauvoei* or tissue extracts were used as templates, only the primer pair designed to produce the 159 bp amplicon gave consistently positive results. No positive results were obtained with any primer pair when purified DNA or biomass from pure cultures of non-target clostridial species were used as templates. Therefore, the PCR primer sets appear to be very specific for identifying *C. chauvoei* in both cultures and tissues.

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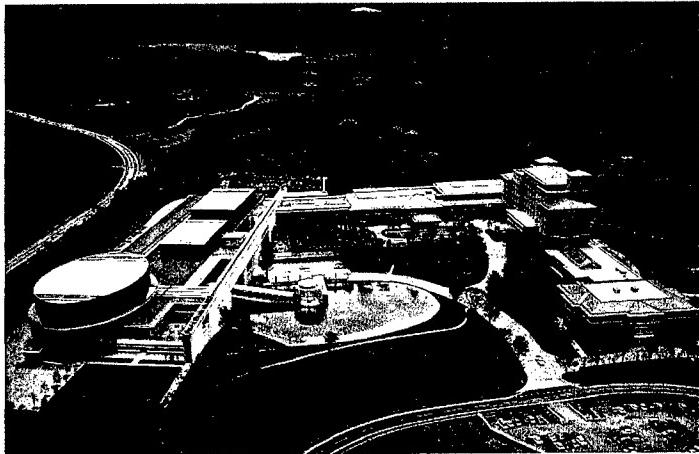
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By Car:

Tokyo-Chiba-Kazusa approx. 80 min.
Narita Airport-Chiba-Kazusa approx. 60 min.

By Train

Tokyo Station-Kisarazu Station 60 min. (Express)
via Tokyo Bay Aqua Line
Tokyo - Kazusa approx. 50 min.
Haneda Airport - Kazusa approx. 30 min.

INFORMATION

Information is also available on line:

<http://w3.ouhsc.edu/cp2000/>

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